HYDROCORTISONE AND A.C.T.H. RELEASE

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It has long been known that the administration of adrenocortical hormones inhibits the secretion of A.C.T.H. by the pars distalis. This has been demonstrated in both chronic and acute experiments. Thus administration of adrenal cortical hormones over a period of days was shown to cause adrenal atrophy (Ingle *et al.*, 1938). In acute experiments Sayers and Sayers (1947), Long (1947) and Gershberg *et al.* (1950) showed that administration of adrenal hormones can prevent the fall in adrenal ascorbic acid which normally follows the injection of adrenaline, histamine, insulin and the application of various operative and other stresses. Moreover, Sayers and Sayers (1948) demonstrated a quantitative relationship between the dose of cortical steroid administered and the degree of inhibition exerted on A.C.T.H. secretion.

The following experiments were designed to determine whether the inhibitory effect of the cortical steroids was exerted directly on the pars distalis. We have used a micro-injector technique to deliver small doses of hydrocortisone directly and continuously to the hypophyseal fossa of rats subjected to a mild stress.

METHODS.

Operative and Micro-Injector Technique. The preparation and use of the micro-injector (Rose and Nelson, 1955) and the detailed technique for the introduction and fixation of a plastic tube into the hypophyseal fossa (H.F.) and the general and endocrine results of such operative interference have been described (Rose and Nelson, 1956).

Index of A.C.T.H. output. We have used adrenal weight as the index of A.C.T.H. output by the pars distalis. This is a most suitable index if one wishes to assess the A.C.T.H. output over an 8-day period. The results are expressed in mg. per 100 gm. of the body weight at the beginning of the experimental period.

The stress. The results from a preliminary experiment are shown in Table 1. Implantation of a tube in the H.F. for 3 days constitutes a stress and the adrenals in this group are significantly larger than normal (compare group B with A). With a tube in the H.F. for 11 days the adrenals do not enlarge further (compare group C with B). However, the additional stress of carrying a 15 gm. micro-injector subcutaneously for the last 8 days of the operative period results in a further increase of adrenal size (compare group D with C and B). We have used the stress of carrying the micro-injector and a unilateral adrenalectomy as a convenient mild stress in the following experiment.

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TABLE 1.

Effect of various operative treatments on the adrenal weight.

Group	Treatment	Number of observations	Mean adrenal weight (mg./100 gm.)
A	Normal	12	15·9±2·8
B	Tube in H.F. for 3 days	16	20·8±1·3
C	Tube in H.F. for 11 days	8	21·0±2·3
D	Tube in H.F. for 11 days and micro-injector subcutaneously for last 8 days	27	25·4±2·2

Experimental design. In order to reduce the number of experimental animals required to obtain statistical significance the left adrenal was removed by operation 3 days after the implantation of the tube in the H.F. At the same time the micro-injector was buried subcutaneously so that it would deliver hydrocortisone for 8 days directly to the H.F. (group E) or elsewhere (group F subcutaneous or lateral to H.F.) as required. The right adrenal was removed at post-mortem 8 days after removing the left adrenal and implanting the micro-injector. The weights of the left and right adrenals from the same animal could thus be compared. Also, at post-morten, the positions of the plastic tube and the side hole in it from which the drug was delivered were checked to make sure they were correctly placed in the H.F.

Preparation and administration of hydrocortisone. Hydrocortisone was administered as a solution containing 50 mg. p.c. of the steroid in an aqueous medium containing 5 p.c. glycerol, 5 p.c. ethanol and M/300 glycine buffered at pH 10. The hydrocortisone was dissolved in the requisite amount of alcohol and then the glycerol and buffer were added. The micro-injector was adjusted so that it would deliver 0·05 to 0·07 ml. per diem. The rats thus received 20–37 μg. hydrocortisone per 100 gm. per day.

RESULTS.

The difference between the weights of the right and left adrenals from the two experimental groups E and F are shown in Table 2. When the hydrocortisone (20–37 μg./100 gm./day) was given subcutaneously or lateral to the

TABLE 2.

Effect of Hydrocortisone on Adrenal Weight.

Group: Treatment: Number of animals:	E Hydrocortisone into H.F. 5	F Hydrocortisone outside H.F. 7
Left adrenal weight (mg./100 gm.)	20·6±3·2*	23·0±6·5*
Right adrenal weight	20·0±3·4	30·6±6·0
Difference between right and left adrenal weight	−·6±0·3†	+7·6±2·62†
Percentage increase of right adrenal over left	−2·9 p.c.	+33·1 p.c.

* Standard deviation

† For the comparison of differences $P < \cdot 01$.

H.F. (group F) the right adrenal was 33·1 p.c. larger than the left. When the same dose range was infused directly into the H.F. the right adrenal was 2·9 p.c. smaller than the left.

In two cases an error was made in the preparation of the micro-injector so that two rats received 48 and 46 μg./100 gm./day into the H.F. The right adrenal was 28 p.c. and 16 p.c. smaller than the left in each case. The results from these two animals were not included in the group subjected to the statistical analysis from which the above conclusions were drawn.

DISCUSSION.

There is no doubt that the blood level of adrenal steroids influences the rate of secretion of A.C.T.H. The primary site of action of the adrenocortical hormones in this respect could be at the hypothalamus or the pars distalis itself. If the primary site of action was at the hypothalamus one could postulate that the decreased A.C.T.H. secretion following adrenocortical hormone administration was due to decreased hypothalamic drive. Thus one could design an experiment whereby hypothalamic drive was eliminated by the administration of adrenal steroids. This would be an ideal experimental animal in which to infuse possible neurohumoral transmitters for A.C.T.H. release into the pars distalis directly. On the other hand, if the adrenal steroids act directly on the pars distalis, inhibition of A.C.T.H. secretion may take place even in the presence of adequate neurohumoral stimulus from the hypothalamus. Prior to these experiments no positive evidence in favour of either site had been presented.

Using a micro-injector technique, we found that hydrocortisone infused continuously and directly into the H.F. produced a greater A.C.T.H. inhibitory effect than the same dose given outside the H.F. These experiments therefore support the view that adrenal steroids act directly at the level of the par distalis to inhibit A.C.T.H. release. They do not eliminate the possibility that the adrenocortical hormones act at the level of the hypothalamus as well as the pars distalis, but this double site of action would seem unlikely.

SUMMARY.

Hydrocortisone at a dose level of 20–37 μg./100 gm./day for 8 days, infused continuously and directly into the hypophyseal fossa of stressed and unilateral adrenalectomized rats results in a significantly greater inhibition of A.C.T.H. output than the same dose given outside the hypophyseal fossa. These experiments support the view that adrenal steroids act directly at the level of the pars distalis to inhibit A.C.T.H. release.

Acknowledgment. The hydrocortisone used in these experiments was kindly presented by Ciba Ltd., Basle.

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CONTROL OF THYROID STIMULATING HORMONE RELEASE

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with a Statistical Analysis by Alison Doig

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The importance of the hypophyseal-portal circulation and the unsolved question of direct innervation of the pars distalis have been the subject of many papers and reviews. We consider that a technique which is capable of delivering drugs directly and continuously to the pars distalis or hypophyseal fossa (HF) might help to elucidate this problem. These and subsequent experiments were designed to study various aspects of the control of anterior pituitary function, using a micro-injector technique previously described (Rose and Nelson, 1955).

METHODS.

Preparation of the Plastic Tubing.

Fig. 1 shows the plastic tube and accessories ready for insertion into the HF. It consists of a polythene tube (A) 0·5 mm. internal diameter which has been drawn out at one end so that it will pass through the lumen of a 26° G. dental needle (B). This tapered polythene tube has an external diameter of approximately 0·5 mm. where it will finally lie in the HF (C). Whilst still hot during the drawing process, the tube is sealed at a point (D) which is just beyond the portion (C) which will lie in the HF. A polyvinyl chloride (PVC) tube (F) 1 mm. internal diameter is threaded over the tapered polythene tube (A) to its maximum diameter. A polythene collar (E) is made with one end expanded by heating and a hole is cut in its side. This is also threaded over the tube (A) as illustrated—it will be noted that the tube (A) enters the collar at the unexpanded end and emerges from the side hole. The polythene tube (A) and the collar (E) are attached to each other by the PVC tube (F) which is tied to both these structures with thread. The expanded end of the collar and the PVC tube can be gripped by threads which will fix the whole structure to the animal's head. A small celluloid washer (G) and a polythene bush (H) are then threaded over the tube (A). The polythene bush is made from 0·5 mm. internal diameter tubing expanded by heating at one end and cut with a slight bevel at the other. The over-all length of the bush is 4 mm. and is necessary to prevent mechanical abrasion and final rupture of the fine polythene tubing where it passes through the skull. A small hole (C) is cut in the side of the tube (A) at such a position that it will finally lie in the HF after the tube is fixed in the skull.

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For rats 200–250 gm. weight the hole should be 1·1 cm. distal to the celluloid washer. The polythene tube (A) is now filled with saline and the proximal end plugged with a short stainless steel wire (I). The plastic tube and accessories are sterilized by immersion in saline at 80° C. for 15 minutes. During this procedure the polythene tube must be under slight tension to prevent distortion. After cooling, the tube (A) is bent to a right angle at its exit from the polythene collar (E). This is simply accomplished by bending the tube and immersing the bend in hot water for a few seconds. The tube and accessories are now ready for implantation.

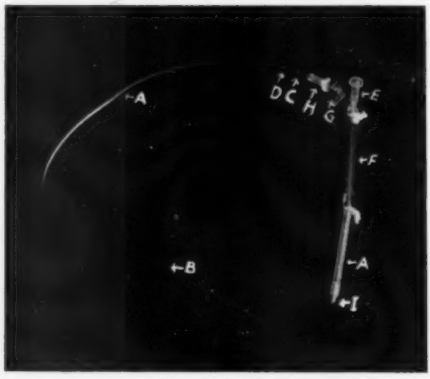


Fig. 1. Plastic tube and accessories ready for insertion into the hypophyseal fossa.
A. polythene tube.
B. hypodermic needle.
C. side hole in polythene tube.
D. sealed portion of tube.
E. polythene collar with expanded end.
F. P.V.C. tube attached to A. and E. with ties.
G. celluloid washer.
H. polythene bush.
I. stainless steel wire plug.

Operative technique.

The operative procedure is carried out in two stages. The first stage consists of insertion and fixation of the plastic tube in the HF. All our operations have been carried out aseptically under open ether anaesthesia. Initially, we attempted to introduce and fix the plastic tube in the HF by the standard parapharangeal approach used for hypophysectomy. This was abandoned because it was technically difficult to fix the tube to the base of the skull. Moreover, we found that introducing a foreign body into the HF by open operation, which exposes the pituitary, leads to more haemorrhage, infection and fibrosis than the present technique. We now use a "blind" temporal approach which does not require the pituitary to be exposed.

A mid line dorsal incision is made along the head, neck and back. The incision extends caudally from the level of the eyes for 4–5 cm. The skin edges are reflected laterally and blunt dissection used to expose the temporal muscle on both sides. The operative field is now ready for the insertion of the plastic tube (A) into the HF. The dental needle (B) is inserted through the right temporal side of the head so that it will traverse the skull and emerge from the same position on the left side. The detailed path of the needle is as follows: (1) It pierces the right temporal muscle at a point just cephalic of the mastoid and just caudal and ventral to the caudal end of the temporal crest. (2) It enters the skull through a gap in the temporaparietal suture. This is the only position in this area where the needle will penetrate through the skull without undue force. The needle should have pierced the temporal muscle at a position just overlying this point. (3) As soon as the needle has entered the skull its point is directed slightly ventrally as it traverses the temporal lobe of the brain. (4) It then passes under the trigeminal nerve. On some occasions the needle actually pierces the nerve. (5) The needle then enters the HF. Usually it can be felt to scrape along the bone making up the floor of the fossa. If the needle has traversed the skull so that its path has been parallel to lines joining the two eyes and the two ears it will lie in the ventracephalic edge of the pars distalis. If the needle is directed cephalically as it enters the skull it can be made to lie just outside the dura making up the dorso-cephalic

surface of the HF. (6) The exit path of the needle is the same as above. Usually, however, the needle strikes the inner table of the skull just ventral to the exit position. This is due to the fact that there is a tendency to direct the needle too ventrally to the HF. If the needle is withdrawn about 0·25 cm. and its point elevated it will then pass easily through the gap in the suture and finally emerge through the temporal muscle on the left side of the head. The bush (H) is then drawn over the dental needle and inserted into the skull by pressure. The dental needle is then drawn completely through the skull and removed from the tube (A). The latter tube is then pulled through the skull until the celluloid washer (G) lies firmly against the temporal muscle. Ties are used to fix the expanded end of the collar (E) and the PVC tube (F) to the temporal muscle (see Fig. 2). The tube (F) is made of PVC because, unlike polythene, it causes a tissue reaction and can be more firmly tied with thread, thus aiding fixation. A rubber disc is threaded over the distal portion of the tube (A) which lies outside the skull and is pushed firmly against the temporal muscle. The tube (A) is cut about 3 cm. from the rubber disc and this length is melted (with a cautery unit)

Fig. 2. Attachment of polythene and P.V.C. tubes to temporal muscle.

Fig. 3. Rubber and plastic disc fixing distal end of polythene tube against temporal muscle.

to form a disc against the rubber and so hold the plastic tube in position (see Fig. 3). The skin is then closed with metal clips. At this stage the plastic tube should traverse the HF with its side hole opening into the pars distalis. Fig. 5 shows such a plastic tube (the proximal part containing indian ink for display) running through the ventrocephalic edge of the HF.

The second stage of the operation consists of implantation of the micro-injector. This was usually carried out 3–4 days after the first stage. The injector was assembled, filled with the drug, the contents sterilized, the water compartment filled and the apparatus weighed as described in a previous paper (Rose and Nelson, 1955). The injector and contents must be warmed to body temperature before implantation into the animal to avoid an immediate delivery of about 0·02–0·05 ml. of the drug, merely due to heat expansion. The old wound was opened and the injector was placed in a subcutaneous pocket made by a previous injection of about 40 ml. of air subcutaneously in the back. The polythene tube leading to the pituitary was tested for patency—often minor blocks could be eliminated by pressure applied with a syringe. No more than 0·02 ml. of fluid should be injected to eliminate a block, otherwise a small haemorrhage may occur. The injector was then connected to the polythene tube (A) and the wound closed with metal clips. Fig. 4 shows a male rat, weighing 220 gm., with a tube fixed in the HF and this tube is connected to a subcutaneously placed injector.

Fig. 4. Rat with tube fixed in HF. The tube is connected to a subcutaneously placed injector.

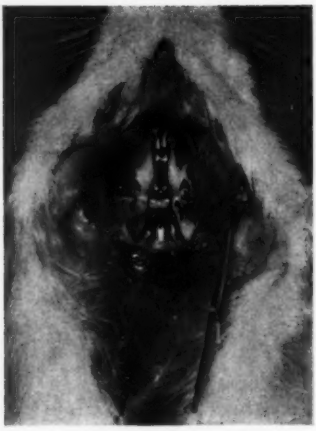


Fig. 5. Polythene tube (the proximal part containing Indian ink for display) passing through the ventro-cephalic edge of the hypophyseal fossa.
A. polythene tube.
T. trigeminal nerve.
HF. hypophyseal fossa.

Care of animals.

Male rats, weighing 180–250 gm., were used. The animals were fed pellets and meat *ad lib.* with free access to water. They were given a post-operative penicillin and streptomycin cover. The animals remained in better condition if they were kept individually in cages—this also allows the food and fluid intake and urine output to be measured. The rats were weighed twice weekly.

Preparation and administration of drugs.

Propyl thiouracil. In preliminary experiments PTU was given as a 0·05 p.c. solution in the drinking water. However, we observed an occasional temporary decrease in fluid intake with emotional and operative disturbance which decreases the goitrogenic response. Consequently all experimental animals received eight daily subcutaneous injections of 40 mg. of PTU made up as an aqueous suspension. The injected area was massaged to disperse the suspension.

Solutions of yohimbine hydrochloride, procaine hydrochloride, atropine sulphate, apresoline (1-hydrazinophthalazine), chlorcyclizine and Regitine (C7337) were prepared by dissolving in saline buffered with M/250 phosphate at pH 4·5. These drugs were administered by the continuous micro-injector technique either into the hypophyseal fossa (HF), just external to the dura making up the dorso-cephalic surface of the HF, or subcutaneously according to the requirements of the experiment. The injectors were designed to deliver by continuous infusion 0·1–0·2 ml. of fluid per diem for 8 days. By varying the concentration of the drug and the volume injected per diem, a range of doses (0·05–4·5 mg./day) could be achieved.

Indices of hypophyseal function.

Organ weights. Thyroid, adrenal, seminal vesicle and prostate weights were measured from immediate post-mortem samples taken at the conclusion of the experiment. The experi-

ments were carried out using adult male rats, which normally would not increase their weight very much over 10 days. However, some of the experimental animals do lose weight, but this is not shared to a significant extent by the endocrine glands. Therefore we have expressed the organ weights in mg. per 100 gm. original rather than final body weight.

Pituitary histology. The pituitaries obtained immediately after the animals were killed at the conclusion of the experiment were fixed in a mixture of formalin (1 part) and saturated mercuric chloride (9 parts) for 5–6 hours. Serial horizontal 7 μ sections of the pituitary were cut and stained with Gomori (1950), periodic acid Schiff (McManus, 1946) and a modification of the Mallory stain (Martin, 1933).

Hypophyseal-portal circulation. In 8 animals the effect of the plastic tube on the hypophyseal-portal circulation was studied. The abdominal aorta was injected with Indian ink, the head removed and fixed in formalin. After decalcification a block of tissue containing the hypothalamus, pituitary and base of skull was embedded in celloidin and sectioned in a sagittal plane at 50 μ. The sections were mounted unstained (Harris, 1950).

Reproductive function in females. Plastic tubes were inserted into the HF in a group of 40 adult female rats. Daily vaginal smears were taken to evaluate the effect of the tube on oestrus cycles.

Posterior pituitary function. Sixty operated animals were placed in individual metabolism cages and the food and fluid input and the urine output were measured daily.

Experimental design.

In Tables 1 and 3 the treatment combinations given to the various groups of rats are shown. The following groups may be described:

(1) Normal controls—group A.

(2) Operated "controls", group B, had micro-injector implanted subcutaneously for 8 days, group C had plastic tube implanted into the HF for 11 days, group D had plastic tube implanted into HF for 11 days and an injector subcutaneously for the last 8 days of the period. This group is referred to as "the double operated controls."

(3) Normal controls given 8 daily subcutaneous injections of PTU—group E. These were killed 24 hours after the last dose of PTU.

(4) Double operated "controls" given 8 daily subcutaneous injections of PTU—group F.

(5) Double operated "controls" given PTU as above but in addition receiving various drugs by the injector technique into the HF, just external to the dura making up the dorso-cephalic surface of the HF, or subcutaneously—groups G to T, excluding P.

(6) Double operated group P given procaine into the HF for 8 days. The procaine infusion was then stopped and with the pituitary tube and injector still *in situ* they were given 8 daily subcutaneous injections of PTU.

At the conclusion of the experimental period the rats were killed by exsanguination whilst under ether anaesthetic. The position of the plastic tube and the side hole in it (Fig. 1c), from which the drug was delivered, was checked to make sure they were correctly placed in the HF.

RESULTS.

General results of operative interference.

We have now operated and implanted tubes into the HF of 450 rats. Injectors were implanted subcutaneously and connected to the tube in 280 of these animals. Twenty-five injectors failed to function. This was due either to a blocked tube, an air bubble over the cellophane membrane or a perforated cellophane. The results of operative interference, implantation of the tube into the HF and implantation of the injector subcutaneously have been assessed from

the entire operated group. However, it should be mentioned that the technique, and therefore the results, have improved with experience.

Mortality. The overall mortality rate was 35 p.c. Most animals died as a result of cerebral haemorrhage at the base of the skull. This occurs as a result of trauma to the cavernous sinus during implantation of the tube into the HF. The animals usually die in the first three days after the operation.

Accuracy of tube implantation. In 6 p.c. of animals post-mortem showed the tubes to be outside the HF. In each case the tube was near the dorso-cephalic surface of the HF. In 12 p.c. of animals the tube was in the HF but the hole in the tube was either to the right or left of the HF.

Infection. Infection along a track around the tube in the HF occurred in 2 p.c. of cases. We have noted that in the presence of infection around the tube the local administration of drugs to the hypophysis always fails to produce the expected pharmacological effect. Infection around the micro-injector occurred in 4 p.c. of cases. The infection was localized by a connective tissue sheath which often contained thick pus. These animals did not show any special deterioration in health other than a slight rise in rectal temperature.

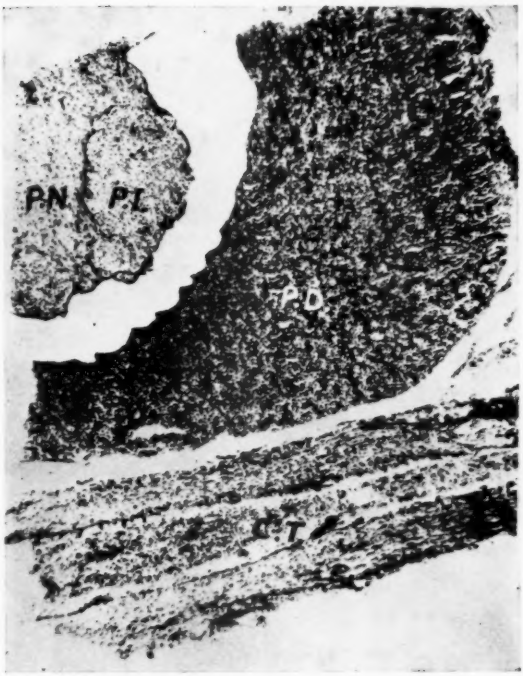
Malalignment of teeth. This has occurred in approximately 10 p.c. of animals. The malalignment is partly due to damage suffered by the jaw joint at the time of operation and partly to pressure of the celluloid washer and rubber disc on the condyles of the mandible which tend to separate the two incisor teeth. Unattended malalignment of teeth results in marked decrease in food intake and consequent loss of weight. This defect can be corrected by suitable clipping of the teeth.

Weight loss. The average body weight lost during the 11-day experimental period was 9 p.c. of the initial body weight. The same loss was observed in those animals where the tube had been outside the HF, thus indicating that the weight loss was not due to hypophyseal damage. There was less weight lost if the animals were kept in individual cages and due attention paid to their teeth. Most animals begin to gain weight after the first fortnight.

Hypothalamic disturbance. Rage reactions lasting 2–3 days occurred in 4 p.c. of animals. We have observed no disturbances in temperature, food and fluid regulation which could be attributed to hypothalamic damage.

Endocrine results of operative interference.

General morphology of pituitary. Infarcts of the pars distalis, resulting in almost complete loss of function, occurred in less than 1 p.c. of cases. The target endocrine glands of these animals were similar to those of a hypophysectomized rat. The usual reaction to the implantation of the tube was a pleomorphic cellular response which gradually became a fibrous tissue sheath surrounding the tube in 10 days (Fig. 6). The operative interference and the presence of the plastic tube did not seem to alter the quantity and detailed histological pattern of the pars distalis.



Figs. 6a and **6b.** Photomicrograph showing connective tissue reaction and position of tube in relation to pituitary. 6a is a horizontal section through the left half of the hypophysis (×70), 6b is a sagittal section through portion of tube and pars distalis (×200).

PD. pars distalis.
PI. pars intermedia.
PN. pars nervosa.
CT. connective tissue around tube.

Fig. 6a.

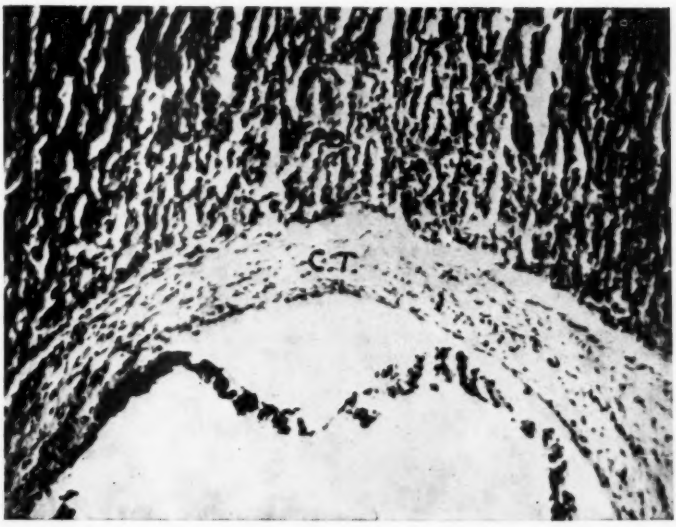


Fig. 6b.

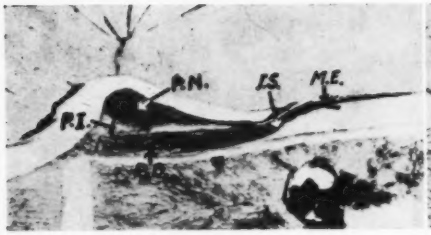


Fig. 7a.

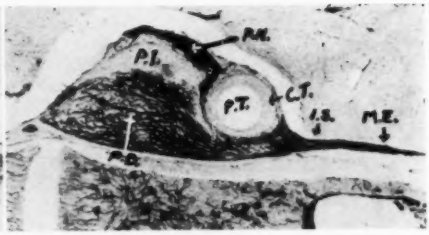


Fig. 7b.

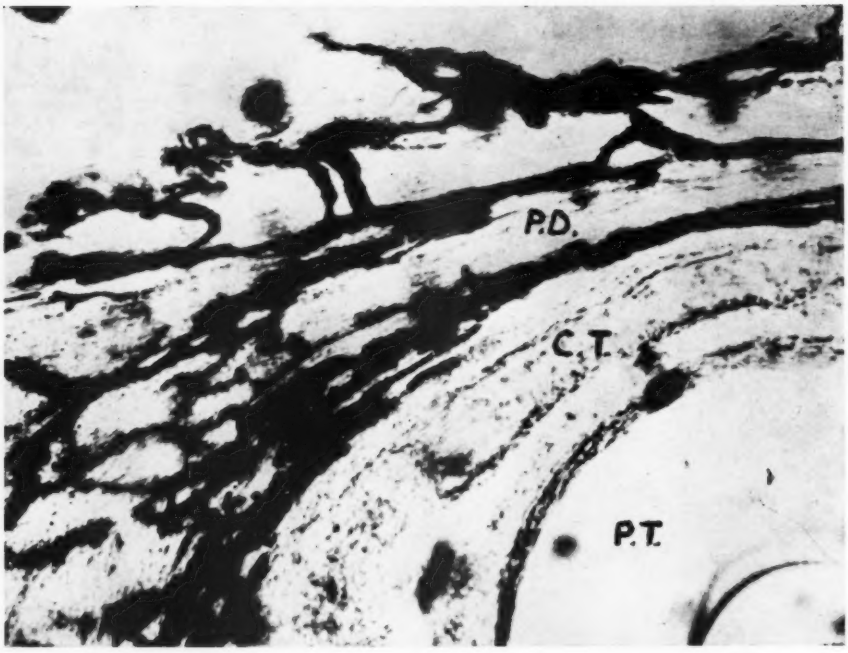


Fig. 7c.

Figs. 7a, 7b and 7c. Photomicrographs of mid-line sagittal sections through hypothalamus, pituitary and base of skull after the vascular system has been perfused with Indian ink. 7a from a normal rat (×8·5), 7b from a rat 8 days after tube implantation, note atrophy of pars nervosa (×8·5). This animal developed diabetes insipidus. 7c from a rat 8 days after tube implantation, showing portal vessels passing around the tube and supplying tissue reaction (×200).

PN. pars nervosa.
PI. pars intermedia.
PD. pars distalis.
IS. infundibular stem.
ME. median eminence.
PT. plastic tube.
CT. connective tissue.

Hypophyseal-portal circulation. Most of our tubes are introduced and finally lie in the ventro-cephalic edge of the HF. This is the area where the stalk enters the gland and so may be liable to damage. Thus diabetes insipidus has been observed in 6 p.c. of our operated and implanted rats. We have not determined how often there has been complete interruption of the portal circulation, but it would seem unlikely that this occurred more frequently than 6 p.c. of cases. Moreover, the portal circulation is capable of regeneration so that complete and persistent interruption would occur less commonly. We have killed animals 5–10 days after implantation and fixation of a plastic tube in the HF to study the regeneration of the portal vessels. Fig. 7a, b and c are photomicrographs of a mid-line sagittal section through the hypothalamus, pituitary and base of skull after the vascular system has been perfused with Indian ink. A transverse section of the plastic tube may be seen in the ventrocephalic edge of the HF. This has been in position *in vivo* for 8 days. The portal vessels are passing around the plastic tube to reach the pars distalis. From the position of the tube in relation to the stalk it is most probable that the injected vessels seen around the tube are made up of both regenerated and original portal vessels. It is not at all surprising that such regeneration does occur when one considers how readily these vessels do regenerate and the difficulty experienced in preventing such regeneration by foreign bodies (Harris, 1950).

The plastic tube becomes surrounded by a cellular and then connective tissue reaction. The blood supply to this tissue may well be derived from the portal circulation which surrounds the tube. This is well illustrated in Fig. 7c, which shows a number of portal vessels passing around the plastic tube and supplying the tissue reaction around it. This special vascular arrangement may assist in allowing a local continuous small application of a drug to influence the entire pars distalis without having as much effect on the posterior lobe.

Posterior pituitary. We have found diabetes insipidus to occur in 6 p.c. of our animals in which a tube has been implanted in the HF. The normal urine output of our animals is 5–10 ml./100 gm./day. Animals with diabetes insipidus excreted 40–70 ml./100 gm./day and the posterior pituitary was found to be atrophic.

Thyroid weights. Table 1 shows the results obtained from rats which have received various operative and PTU treatment combinations and also for the composite groups X and Y, which are defined subsequently. The results are given as the mean and standard deviation of each group, and, for purpose of analysis, a logarithmic transformation is used to bring the data nearer to normality.

An analysis of variance of groups A, B, C, D showed that the presence of the tube and/or injector did not have any significant effect on the normal thyroid weights. The four groups were pooled to give joint estimates of the mean thyroid weight for normal rats and the standard deviation of a single observation. This composite group is designated X.

TABLE 1.

Effect of operative and PTU treatment combinations on thyroid and adrenal weights.

Group	Drug and operative treatment	No. of Animals	Degrees of freedom	Thyroid weights	
				Working units (logarithms)	Retransformed to original units (mg./100 gm.)
A	Normal	18	17	0·946±0·080*	8·83±1·20*
B	Injector Subcutaneously	6	5	0·890±0·078	7·76±1·20
C	Tube in HF	8	7	0·877±0·045	7·53±1·11
D	Double Operated	15	14	0·915±0·081	8·22±1·21
E	P.T.U. Treated	14	13	1·167±0·068	14·69±1·17
F	Double Operated P.T.U. Treated	17	16	1·147±0·116	14·03±1·31
X	Pooled Results from Groups A-D	47	30·6 (effective)	0·906±0·064	8·05±1·16
Y	Pooled Results from Groups E & F	31	22·9 (effective)	1·161±0·090	14·50±1·23
				Adrenal weights	
				Working units	Retransformed
A	Normal	12	11	1·20±0·080	15·7±1·20
C	Tube in HF	9	8	1·32±0·083	20·7±1·21
D	Double Operated	27	26	1·40±0·090	25·1±1·23

* Standard Deviation.

Subcutaneous administration of PTU results in a highly significant increase in thyroid weight. Normal rats (group E) and double operated rats (group F) received daily PTU injections. Means and variances from groups E and F were compared and were found not to differ significantly. Thus the operative trauma and stress, fixation of a tube in the HF and an injector subcutaneously did not prevent the increased TSH release and consequent development of a goitre following PTU administration. The results from these two groups were pooled to give the composite group Y.

Adrenal weights. Table 1 shows the results obtained from various treatment combinations. One could expect the stress of the entire experimental procedure to result in larger adrenals. This supposition was confirmed by the ordinary t-test which showed that group C (tube in HF) and D (double operation) both differed significantly from normal group A. The adrenals from group D appeared on the ordinary t-test to be significantly larger than those from group C. This test is, however, inappropriate for this ocasion and when a more suitable test was applied the difference in means, though large, was no longer significant. (Details of this harsher test are given subsequently.) The results thus show that operative interference to the pars distalis and the presence

of a plastic tube in the gland do not prevent the increased release of ACTH under stress.

Reproductive function. The response of the seminal vesicles and the prostate to different treatments may be expected to be closely linked. Therefore a score based on the sets of readings for both organs was calculated, using the standard technique for calculating a single bivariate discriminant function between several groups. The coefficients of the discriminant function were chosen so that maximum over-all discrimination was obtained and the scale was chosen to give an estimated variance of unity for the score obtained from a single animal. Denoted by X_{SV} and by X_P, the logarithm of the weight (mg./100 gm. body weight) of the seminal vesicles and prostate, respectively, the discriminant function is $d = \cdot 876\, X_{sv} + 7 \cdot 98\, X_P$.

All further steps were carried out using d values as if they were normal variables with unit estimated variance based on 55 degrees of freedom. (Some of the results on which this is based are not included in this paper.) Table 2

TABLE 2.

Effect of operative treatment combinations on seminal vesicle and prostate weights.

Group	No. of animals	Treatment	$\bar{X}_{sv}$	$\bar{X}_P$	d
A	12	Nil	2·647	2·104	19·11
B	6	Injector subcutaneously	2·534	2·209	19·85
C	4	Tube in HF	2·361	2·037	18·33
D	15	Double operated	2·424	2·113	18·99
G	2	Double operated and Yohimbine (greater than 0·7 mg./day into HF)	2·318	2·336	20·67
M	8	Double operated and Procaine (greater than 0·8 mg./day into HF)	2·205	2·052	18·31
X	37	Composite group A+B+C+D	2·507	2·117	19·09

$\bar{X}_{sv}$ and $\bar{X}_P$ = the mean logarithm of the seminal vesicle and prostate weights.
d = degrees of freedom.
Animals in group G and M received into the hypophyseal fossa yohimbine (0·97·2 mg./day) and procaine (1·1-4·5 mg./day) respectively.

shows the results obtained from rats with various treatment combinations. The analysis showed that there was no significant difference between any of the four groups A, B, C and D, and they were pooled to give X, which is also shown in Table 2. Thus the presence of a tube in the HF has not resulted in any significant decrease in seminal vesicle and prostate weight during the experimental period.

Thirty female adult rats were operated and a tube implanted into the HF. One rat remained in anoestrus for 3 weeks, 5 rats developed irregular cycles and the remainder showed regular oestrus cycles usually preceded by a pause or irregularity for 2–5 days. The results from both male and female rats indicate that there is not much depression of gonadotrophic hormone production in most animals by the operative stress, etc. This is not surprising since it is known

that after incomplete hypophysectomy 30 p.c. of anterior pituitary tissue may be sufficient to maintain normal sex function and as little as 10 p.c. may maintain some ovarian activity (Smith, 1932). Serial sections through the pars distalis do not show gross damage to that organ.

Endocrine effect of drug infusion into the HF.

Organ weights. Table 3 shows the thyroid and adrenal results from various groups all of which had received PTU by subcutaneous injection. Y is a composite group made by pooling E (normal rats receiving PTU) and F (double operated rats receiving PTU) and therefore gives a joint estimate of the mean goitrogenic response for normal and operated rats and the standard deviation

TABLE 3.

Effect of drug treatment on the goitrogenic effect of PTU and the adrenal response to the accompanying operative stress.

Group	No. of animals	Degrees of freedom	Treatment			Thyroid weights	
			Drug	Site of Administration	Dose mg./day	Working units (logarithms)	Retransformed to original units (mg./100 gm.)
X	31	22·9 effective	nil	—	—	1·161±0·090	14·50±1·23
G	6	5	Y	H.F.	>0·7	0·898±0·093	7·91±1·24
H	7	6	Y	O.H.F.	>0·7	1·286±0·128	19·32±1·34
I	5	4	Y	S.C.	>0·7	1·121±0·097	13·21±1·25
J	5	4	Y	H.F.	<0·7	1·187±0·098	15·37±1·25
K	3	2	Y	O.H.F.	<0·7	1·277±0·043	18·97±1·10
L	5	4	Y	S.C.	<0·7	1·095±0·086	12·45±1·22
M	10	9	P	H.F.	>0·8	0·863±0·074	7·30±1·19
N	2	1	P	H.F.	<0·8	1·141±0·097	13·84±1·25
O	1	0	P	O.H.F.	<0·8	1·182	15·20
P*	3	2	P	H.F.	>0·8	1·272±0·013	18·71±1·03
Q	3	2	C	H.F.	1·0	1·152±0·252	14·19±1·78
R	2	1	AP	H.F.	0·2+0·7	1·196±0·165	15·70±1·46
S	3	2	R	H.F.	0·6-1	1·206±0·101	16·07±1·26
T	3	2	AT	H.F.	0·6-1·0	1·228±0·136	16·90±1·37
						Adrenal weights	
						Working units	Retransformed
G	4	3	Y	H.F.	0·7	1·372±0·066	23·55±1·16
M	9	8	P	H.F.	0·8	1·354±0·107	22·59±1·28

Y = yohombine AP = apresoline HF = hypophyseal fossa
P = procaine AT = atropine OHF = just outside hypophyseal fossa
C = chlorcyclizine R = Regitine SC = subcutaneous
* Procaine infusion stopped and PTU treatment commenced 8 days prior to conclusion of experiment.

of a single response. The other groups received various additional drugs delivered by the micro-injector technique into the hypophyseal fossa (HF), just outside the fossa or subcutaneously. The effect of such drug treatments on the goitrogenic response to PTU was estimated by comparing the particular group with Y. If a series of comparisons are examined using the t-test then, by chance alone, a certain proportion of the results will appear to be significant. To reduce the probability of drawing erroneous conclusions as a consequent of these spurious "significances" a harsher test was applied whenever several t-tests were carried out. Application of this stricter test will not have any effect on results already shown to be non-significant. Hence we need only to examine the t-tests which are significant. The comparisons shown in the first column of Table 4 require further investigation. In all, 14 comparisons have been made and theory shows (Scheffe, 1953) that the appropriate statistic to compute for each case is $F_{14\nu}=\frac{t^2}{14}$

where ν is the number of degrees of freedom given in the third column of Table 4. On this test the only two treatments giving mean thyroid weights which differ significantly from Y are G and M.

TABLE 4.

Tests for significance comparing mean logarithm of thyroid weights of treated groups ($\bar{X}_i$) with those of pooled control group ($\bar{X}_Y$).

Comparison Group i—Group Y	$\bar{X}_i-\bar{X}_Y$	d.f.(ν)	$\sqrt{S^2\left(\frac{1}{n_i}+\frac{1}{n_Y}\right)}$	t_ν	$F_{14\nu}$
G-Y	0·2632	28	0·03717	7·08	3·6
H-Y	0·1249	29	0·03921	3·19	<1
K-Y	0·1161	25	0·04681	2·48	<1
M-Y	0·2983	32	0·02912	10·24	7·5
Q-Y	0·1108	25	0·05165	2·4	<1

Groups G and J received yohimbine and groups M and N procaine directly into the hypophyseal fossa. A regression analysis was carried out and a linear dose relationship between the log dose and the log response was demonstrated for each drug, as seen in Fig. 8a and b.

The following summary details the effect of various drug treatments on the goitrogenic response to PTU:

(1) Yohimbine infused directly into the HF at a dose level greater than 0·7 mg./day (dose range 0·97 to 2 mg./day) prevents the normal goitrogenic response to PTU (compare group G with Y). Lower doses of yohimbine (dose range 0·08 to 0·35 mg./day) are not effective (compare group J with Y). A regression analysis shows a linear dose response relationship for this drug. Yohimbine in similar doses infused just outside the hypophyseal fossa or sub-

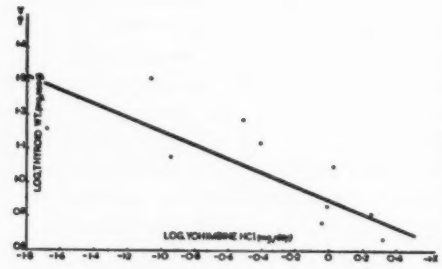


Fig. 8a. Regression line relating logarithm of dose of yohimbine Y with logarithm of thyroid weight X. (Y=·953—·202 X).

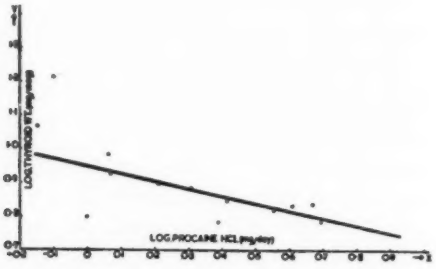


Fig. 8b. Corresponding regression line for procaine (Y=·985—·321 X).

cutaneously does not prevent the usual goitre from developing (compare H I K with Y).

(2) A similar goitrogenic blocking effect was also obtained by direct infusion of procaine into the HF (dose range 1·1 to 4·5 mg./day, compare group M with Y). Lower doses (0·7 mg.) were not effective (compare group N with Y). A regression analysis also shows a linear dose response relationship for procaine. When procaine was given outside the HF most rats died. One animal received 0·16 mg. of procaine per day outside the fossa and developed a goitre. Other rats receiving higher doses of procaine outside the HF died and some of these were already developing a goitre after 4–5 days PTU treatment.

(3) One group of rats (P) was given procaine (in an effective goitrogenic blocking dose, range 1·1–1·4 mg. per day) directly into the HF for a period of 8 days, during which time they did not receive PTU. At the end of the 8-day period the procaine infusion was stopped, the implanted tube in the HF and micro-injector remained *in situ* and the animals received daily PTU injections for 8 days. These animals developed a goitre which was slightly larger than the control goitre group Y. The difference was significant by the t-test but was not significant when the harsher test was applied. (Compare group P with Y.) The results from this experiment indicate that the changes produced by the prolonged infusion of procaine (in adequate goitrogenic blocking doses) into the hypophysis are reversible.

(4) No goitrogenic blocking action could be demonstrated in various small groups of rats by the direct infusion of chlorcyclizine (dose 1 mg./day—compare group Q with Y), apresoline (dose 0·2–0·7 mg./day—compare group R with Y), Regitine (dose 0·6–1 mg.—compare group S with Y) and atropine (dose 0·6–1 mg./day—compare group T with Y). Larger doses of apresoline and Regitine could not be given into the HF because they were invariably fatal.

(5) Groups G and M had received an adequate dose of yohimbine and procaine directly into the HF to block the goitrogenic response to PTU. It was

of interest to determine what effect this drug treatment on the hypophysis would have on other endocrine glands.

In a previous section of this paper it was shown that the entire operative and implantation technique was sufficiently stressful to produce a considerable increase in adrenal weight (compare double operated group D with normal group A). The difference in means between D and each of G and M was compared by the ordinary t-test and found to be not significant. The results indicate that the goitrogenic blocking doses of yohimbine and procaine infused into the HF do not prevent the increased release of ACTH and consequent adrenal enlargement with the operative stress stimulus. This has been confirmed in a further group of 3 rats receiving PTU. The left adrenal was operatively removed and weighed immediately prior to the procaine infusion (1·4–1·6 mg./day) into the HF. Eight days later the animals were killed. The goitrogenic action of the PTU was blocked (mean thyroid weight 8·4 mg./100 gm.), but the remaining right adrenal was 28 p.c. larger than the left which had been removed prior to the procaine infusion.

For seminal vesicle and prostate, an analysis of the results presented in Table 2 shows that groups G and M, when tested against X, gave values of t exceeding the 95 p.c. limit. On applying the harsher test the values obtained were not significant.

Thus the possibility that procaine, but not yohimbine, in goitrogenic blocking doses, when infused into the HF, may depress the gonadotrophic hormone production cannot be excluded. However, histological examination of pituitaries infused with yohimbine or procaine show numerous and active F.S.H. and L.H. cells.

Pituitary histology. The histological data from pituitaries infused with procaine and yohimbine is still preliminary and certain aspects only will be described. The pars distalis appears to be healthy—thus features of toxic degeneration, such as cloudy swelling, etc., were not observed. The many active macrophages picking up haemoglobin is also evidence for good function. A variable degree of tissue reaction had developed around the polythene tube and small areas of infarction and fibrosis were often found, but in most cases the total quantity, general and histological architecture were not significantly altered.

Gomori positive cells were absent from pituitaries of rats which had received PTU, irrespective of whether they had received goitrogenic blocking doses of procaine or yohimbine into the HF or not. The disappearance of Gomori positive granules from the thyrotrophs is related to the discharge of the stored TSH in these cells (Purves and Griesbach, 1951b). Thus goitrogenic blocking doses of yohimbine and procaine infused into the HF do not prevent the initial discharge of stored TSH in response to the PTU treatment which was begun 24 hours prior to the drug infusion into the HF. This result is similar to that obtained

by Bogdanove and Halmi, who found that lesions in the hypothalamus which prevented the goitrogenic response to PTU did not prevent the initial discharge of stored TSH and consequent loss of Gomori positive granules in the thyrotrophs (Bogdanove and Halmi, 1953).

Purves and Griesbach (1951a) studied the development of thyroidectomy cells following thyroidectomy or PTU administration. The results of Bogdanove and Halmi suggest that less thyroidectomy cells develop in those rats with hypothalamic lesions which do not develop a goitre with PTU administration (1953). However, it is not possible to say whether goitrogenic blocking doses of procaine or yohimbine infused into the HF prevent the development of these thyroidectomy cells because of the short (9-day) duration of our experimental period.

Posterior pituitary function. Rats in groups G and M who had received goitrogenic doses of yohimbine and procaine did not develop diabetes insipidus. The normal urine output of our animals is 5–10 ml./100 gm./day. Animals with diabetes insipidus excrete 40–70 ml./100 gm./day. Animals in groups G and M excreted slightly less urine per diem than normal—this is probably the result of operative and emotional stress on fluid intake.

DISCUSSION.

The value and use of the micro-injector technique to study control of hypophyseal function.

We have applied a new micro-injector technique to the problem of hypothalamic control of hypophyseal function. The details of construction and use of the injector have been previously described (Rose and Nelson, 1955). The essential features of the technique are: (a) The injector can be buried subcutaneously and is therefore carried about by the animal. (b) It can deliver drugs at a relatively constant rate for many weeks. (c) The drugs may be given directly to any desired situation *via* a previously implanted tube. (d) If a suitable dose of drug is chosen it may be possible to produce a prolonged local pharmacological action in one area of the body, e.g., pars distalis, without an appreciable effect on the general metabolic status.

The hypothesis that the hypothalamus controls the pars distalis and the mechanism by which the control is exerted has been a most controversial issue for some time. The recent papers of Harris (1948, 1952, 1955) and Zuckerman (1955) summarize the problem and allow most of the literature on this subject to be obtained from primary and secondary sources. An extensive survey of the literature is therefore not necessary, but a brief outline of the design and interpretation of the experimental methods which have been used in this field will form a basis for discussion as to the value, scope and use of the new micro-injector technique to the problem.

(1) The normal anatomical pathway, direction of flow and ability of the

hypophyseal-portal vessels to regenerate after section have been studied and well recorded. The presence or absence of secretory nerves to the pars distalis remains a controversial issue. The problem centres around the histological differentiation of nerves and reticular fibres by special stains. Until this is solved the anatomical pathway and ability of the alleged nerves to regenerate will also remain unanswered.

(2) Stimulation or destruction of discrete areas in the hypothalamus. These could stimulate or destroy a pathway or group of cells necessary for the humoral or alleged nervous transmission to the pars distalis.

(3) Section of the stalk cuts both the portal vessels and alleged nerves and both could possibly regenerate.

(4) Section of the stalk with techniques (paper plates, etc.) devised to prevent regeneration of the portal vessels would also prevent regeneration of the alleged nerves. Moreover, a variable degree of infarction and fibrosis occurs in the pars distalis after such a procedure and it is difficult to correlate quantitatively the degree of pituitary dysfunction with ischaemic damage and loss of specific humoral and/or nervous influences.

(5) Transplanting the pituitary away from its normal situation cuts both the portal circulation and alleged nerves. This technique suffers from the disadvantage that the transplant must take and revascularise in a new situation. Most workers agree that the transplanted pituitary loses its specific differentiated cellular pattern and has a very limited function. However, these experiments do not give any evidence to answer the critical question as to whether the change in the transplanted gland is due to loss of specific humoral and/or nervous influences.

(6) Pharmacological experiments involving the systemic administration of "neurohumoral transmittors" and their antagonists have occupied the attention of many workers. When positive results are obtained the question always arises as to the primary site and mode of action, and the influence of systemic side effects on the response. When a negative result is obtained it may be due to the fact that the drug has not reached the proposed site of action in adequate concentrations to be effective. To avoid the above difficulties drugs have been given as a single application directly to the hypothalamus, intact pituitary and transplanted gland. This resembles the direct approach (local application or intra-arterial injection) used to study possible humoral factors in the transmission of impulses at ganglia and neuromuscular junctions, etc. However, this technique is more easily applicable to the latter experiments because anaesthesia and operative stress to the animal, and isolation and exposure of the experimental site does not unduly interfere and the response becomes measurable very soon after the stimulus. Unfortunately these advantageous conditions are not inherent in the study of the hypophyseal problem. Moreover, when a local single injection is given a non-specific response may be recorded, e.g., a small dose of adrenaline

injected into the anterior chamber of the eye containing a transplanted pituitary causes release of ACTH. Some workers feel that this may be due to pituitary damage with non-specific release of ACTH. Prolonged injection with a prolonged response does not present this difficulty in the interpretation of the results.

From a consideration of sections 2, 3, 4 and 5 of the above discussion, it is apparent that the critical experimental difficulty has been to eliminate independently the influence of either the portal circulation or the alleged nerves leaving the other intact. Moreover, this should be attained if possible without ischaemic damage to the pars distalis. We have made an attempt to achieve this experimental design by using the micro-injector technique to infuse local anaesthetics into the HF. We argued that it might be possible to block conduction in the alleged nerve terminals of the pars distalis without gross damage to the portal circulation or the gland. However, drugs which are capable of blocking nerve conduction produce other pharmacological and metabolic distortions in both nerve and other tissues. The specificity of results obtained with local anaesthetics may be enhanced by (a) demonstrating a discrete and reversible alteration in pituitary function, (b) using a wide range of local anaesthetics with varying chemical structures and side effects, (c) using drugs which produce some of the side effects of local anaesthetics but which themselves do not block nerves.

From a consideration of section 6 of the above summary, it will be appreciated that the critical pharmacological difficulty has been to determine the primary site of action of the "neurohumoral transmittors" and their antagonists. Moreover, and in particular if the drug is unstable, the question arises as to whether it has reached the proposed site of action in adequate concentrations to be effective. If the hypothalamus controls the pars distalis by humoral factors, which must be delivered directly to the gland *via* the portal circulation, one might logically infer that direct infusion into the gland would be the desired experimental technique. The pharmacological design and interpretation of such experiments will be similar, but more difficult, than the corresponding problem of neurohumoral transmission at ganglia, etc. The evidence in favour of a particular transmittor would depend upon:

(a) Isolating the humoral substance in the hypophyseal-portal blood. Technically this would be extremely difficult.

(b) Altering pituitary function by continuous and direct application of drugs which block the transmittor or the enzyme which destroys it. Unfortunately, these blocking drugs are by no means specific and there is a good deal of overlap in their action.

(c) Applying the "transmittor" substance continuously and directly to the pituitary to mimic the normal humoral control. However, the pituitary in its normal situation may be receiving from the hypothalamus sufficient of the "transmittor" for maximal function and therefore may not respond to an applied

excess. Application of the transmittor to the transplanted gland would therefore be more likely to produce positive results.

Discussion of present experimental results.

In the following discussion an attempt will be made to analyze the mechanism, under our experimental conditions, of the goitrogenic blocking effect of yohimbine and procaine.

(1) Mechanical trauma by the introduction of a fixation of the plastic tube in the HF could possibly result in impaired pituitary function. Critical structures which might be damaged are the pars distalis, hypophyseal portal circulation or the alleged nerves from the hypothalamus to the pars distalis. However, the histological results generally do not show gross damage to the pituitary. A connective tissue reaction around the tube and small infarcts are commonly seen but in most cases damage is minimal and would not interfere with pituitary function. Diabetes insipidus occurred in only 6 p.c. of our operated animals. This can be taken as an index of how often the stalk, and, consequently, the hypophyseal portal circulation and alleged nerves to the pars distalis, would be damaged. Sagittal section of the hypothalamic pituitary area after perfusion of the vascular tree with Indian ink shows a profuse set of vessels passing from the median eminence around the tube to the pars distalis. Even if the operative introduction of the plastic tube did damage the portal vessels, adequate regeneration has occurred. It is a common experience after incomplete hypophysectomy to find that even small remnants of pituitary tissue are capable of producing sufficient excess TSH to produce a goitre when the animal is given PTU. Finally, introduction and fixation of a tube in the HF does not prevent the usual adrenal enlargement of stress or the goitre development of PTU administration.

(2) Greer (1951, 1952) and Bogdanove and Halmi (1953) showed that hypothalamic lesions in the rat could partially block the goitrogenic response to PTU. These workers chose localized hypothalamic lesions as the method of disrupting the hypothalamic-hypophyseal relationship, because discrete disturbances of pituitary function could be obtained without grossly impairing the nutritive and special vascular supply of the gland. Since yohimbine and procaine are both capable of blocking conduction, and, with the above results in mind, the question arises as to whether these drugs have leaked out of the HF, disturbed hypothalamic function and so impaired the TSH release from the pars distalis. Evidence is available to indicate that some of the drugs may leak out of the HF. Thus the infusion of 5 p.c. procaine or 1–2 p.c. Regitine into the HF kills most of the experimental animals, presumably because the drug has leaked out of the fossa and interfered with brain function. In the present experiments the volume per diem injected into the HF was 0·1 to 0·2 ml., which represents 15–30 times the volume of the gland. It is possible that if

the drugs were delivered to the HF in a smaller volume less would escape. The quantity and spatial distribution of the drug outside the HF has not been studied, but it seems probable that some reaches and circulates in the cerebro-spinal fluid. Many drugs, e.g., adrenaline, are only slowly absorbed from and destroyed in the C.S.F. (Becht, 1920). This is also true for procaine. Thus most tissues and serum, but not cerebro-spinal fluid (Kalow, 1952) contain a procaine esterase. When procaine is injected into the cerebro-spinal fluid it it hydrolyzed about 100 times slower than in plasma and is only slowly absorbed. Therefore even small amounts of procaine which might leak into the cerebro-spinal fluid could accumulate and affect nervous tissue. However, it is most unlikely that escape of procaine and yohimbine to nervous centres can explain their goitre-blocking action. Thus yohimbine (at a goitrogenic blocking dose when given into the HF) is not effective in blocking a goitre when given outside the HF. In these animals the tube was placed just outside and along the dorso-cephalic surface of the fossa and under the ventral surface of the hypothalamus. When procaine was injected outside the fossa most of the animals died. Some of these latter were beginning to show a goitre response when they died 4 days after they had received PTU. Bogdanove and Halmi (1953) describe well-known changes in behaviour, etc., with lesions in the hypothalamus. We have not observed rage reactions, hyperphagia, hyperthermia, diabetes insipidus or failure to secrete ACTH with stress, whilst the rats were receiving procaine or yohimbine in or out of the HF.

(3) Brown-Grant *et al.* (1954a, b), using the rate of release of I^{131} from the thyroid gland of rabbits as an index of endogenous TSH release, showed that emotional and physical stress which is known to increase ACTH production results in a decreased secretion of TSH. In the present experiments we found that operative introduction and fixation of a tube in the HF and implantation of an injector subcutaneously causes a marked enlargement of the adrenals. In view of the above, the question naturally arises whether our operated and stressed rats were unable to increase their TSH production when given PTU. However, rats subjected to the double operation develop the same goitre as controls. Moreover, rats injected with procaine or yohimbine not developing a goitre did not have larger adrenals than the control operated group. Recently we have studied the release of I^{131} from the thyroid gland of rats which have been subjected to various forms of stress (immobilization or double implantation operation) and have shown that a continued stress does not produce a continued inhibition of TSH secretion after 1–2 days (Rose and Nelson, 1955).

Thus the stress of the double operation and/or the infusion of yohimbine or procaine into the HF cannot be the explanation for the goitre-blocking action of these drugs.

(4) The goitrogenic-blocking effect of yohimbine and procaine must be related to their drug action on the pars distalis or the alleged nerve terminals

in the gland. The elucidation of the mode of action of these drugs in blocking the goitre response could give valuable clues as to the controlling mechanism of the pars distalis.

(a) Yohimbine or procaine produces marked alteration in the metabolism of both neural (Watt, 1949; Toman, 1952) and other tissues. Thus Goodman and Gillman (1941) state that procaine is a depressant for all peripheral excitable tissues, and Sollman and Estable (1948) suggest that procaine may be more effective in depressing skeletal muscle than nerve. The metabolic effects of yohimbine have been studied by Schmidt (1936), Doty and Gerard (1950) and Bradley (1951) and need not be discussed in this paper. Our results, however, do not suggest that these drugs are acting merely in a non-specific fashion to depress the pars distalis. Thus not all functions of the pars distalis are depressed. The pituitary is capable of increased secretion of ACTH as a response to stress. Numerous and active gonadotrophs can be seen in the histological sections of the pituitary even though there is a small but statistically insignificant decrease in the weights of the seminal vesicle and prostate. The pituitary looks healthy and functioning—active macrophages may be seen which have picked up haemoglobin, and there is an abundance of active gonadotrophs and perhaps stimulated acidophils (the histological data are still preliminary). Moreover, whatever changes procaine produces in the pars distalis are readily reversible. This was illustrated by the experiment whereby after 8 days of procaine infusion into the HF, PTU given daily by subcutaneous injection produced a normal goitre.

(b) Yohimbine and procaine (Toman, 1952) are both capable of blocking nerve conduction. However, this is by no means their only action, so that our results are compatible with, but do not prove, the hypothesis that secretory nerves to the pars distalis are necessary for the increased TSH discharge following treatment with PTU and that yohimbine and procaine act by blocking these nerves.

(c) Yohimbine and procaine could also act by blocking "neurohumoral" transmittors reaching the pars distalis *via* the hypophyseal portal circulation. However, the neurohumoral antagonists are not specific and a great deal of overlap occurs. The concept was suggested by Burn and Dutta (1948) that acetylcholine, adrenaline, histamine and probably others were related neurohumoral hormones and these were blocked by common antagonists. Thus the vasoconstrictor action of adrenaline on the vessels of the rabbit's ear can be abolished by procaine, atropine, Regitine, ergotamine, dibenamine and various antihistamines. The large overlap and general non-specificity of these drugs has been confirmed by other workers. There are many exceptions to the rule that the true specific antagonist acts at a lower concentration and "sticks" to the receptor site longer and more firmly (Burn and Dutta, 1948). Moreover, the results obtained from one site of action cannot always be applied to another. Thus, for

a number of compounds, there was no correlation between their ability to block the pressor and glycaemic response to adrenaline (Harvey *et al.*, 1952). It is no wonder that a pharmacological interpretation of our results is indeed very difficult. However, the results obtained with yohimbine, procaine, and for a small number of animals, Regitine, apresoline, atropine and chlorcyclizine, suggest that adrenaline, nor-adrenaline, acetyl-choline and histamine are not the chemical mediators for TSH release. An interesting possibility to be considered is serotonin. This substance has recently been studied as a possible neurohumoral transmittor in the central nervous system. The structure of yohimbine suggests that it may be a serotonin antagonist and this has been confirmed by experiment (Shaw and Woolley, 1953). Local anaesthetics (Sinha and West, 1953) also block the action of serotonin, but apresoline, Regitine and antihistamines are only very poor antagonists to serotonin (Page and McCubbin, 1953).

SUMMARY.

A new micro-injector technique has been applied to the problem of hypothalamic control of the pars distalis. The general and endocrine effects of the operative stress and traumatic damage to the pituitary by the experimental procedure have been described. A brief outline of the design and interpretation of the experimental methods which have been used to study the hypothalamic-hypophyseal problem is given and forms a basis for discussion as to the value, scope and use of the micro-injector technique in this problem.

Using the micro-injector technique, various blocking drugs have been continuously injected for 8 days directly into the hypophyseal fossa, just outside the fossa or subcutaneously. The effect of such drug treatment on the goitrogenic response to subcutaneous propyl-thiouracil administration has been studied.

Yohimbine or procaine injected directly into the hypophyseal fossa can prevent the usual goitre development with propyl-thiouracil administration. A regression analysis showed a linear dose response relationship for these drugs.

Yohimbine or procaine, in goitre-blocking doses, injected into the hypophyseal fossa, does not prevent the continued increased ACTH discharge and consequent enlarged adrenals which accompany stress. Procaine infusion results in a small but statistically non-significant decrease in the weights of the seminal vesicle and prostate.

Yohimbine, in goitre-blocking doses, given just outside the hypophyseal fossa or subcutaneously, does not prevent the development of a goitre. Procaine given just outside the fossa usually kills the animals but goitres are already beginning to develop if the animals live 4–5 days.

Procaine, in a goitre-blocking dose, given into the hypophyseal fossa for 8 days, does not prevent the pituitary from releasing TSH and producing a goitre if propyl-thiouracil is given after the procaine infusion has ceased.

Preliminary histological examination of sections of pituitary which had received goitrogenic-blocking doses of procaine or yohimbine has been carried out. The pars distalis looks healthy and functioning, there is no significant decrease in the quantity of pituitary tissue and no gross alteration in the histological pattern.

A goitrogenic-blocking action could not be demonstrated in small groups of rats by the continuous infusion of atropine, chlorcyclizine, Regitine or apresoline into the hypophyseal fossa.

The pharmacological significance of these results in relation to the problem of the control of the pars distalis is discussed.

Acknowledgments. We are grateful to Miss H. Wischusen and Dr. R. Maynard, of the Pathology Department, Heidelberg Repatriation Hospital, for the preparation of the histological material. We would also like to express our thanks to Dr. W. E. Griesbach, of the Thyroid Research Medical School, Dunedin, for preparation of, and comments on, some of the pituitary sections.

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GOITROGENIC ACTION OF PROPYL-THIOURACIL

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The striking goitrogenic properties of thiouracil and its derivatives are generally attributed to an increased secretion of thyrotrophic hormone (TSH) following inhibition of thyroid hormone production. It has been suggested that thiouracil may also act by augmenting the effect of TSH. The purpose of these investigations was to study this latter proposed action of the goitrogen by injecting a small dose of propyl-thiouracil (PTU) into one thyroid lobe.

METHOD.

The experiment was carried out on 3 mongrel dogs. Using ether-chloroform anaesthesia, complete surgical asepsis and a mid-line ventral incision in the neck, the thyroid lobes were isolated. A polythene tube was inserted into each thyroid lobe. This was achieved by transfixing the thyroid lobe in its longitudinal axis with a 21G hypodermic needle through which the polythene tube was threaded. The needle was then withdrawn leaving the polythene tube in the thyroid. The preparation and sterilization of the plastic tube have been previously described (Rose and Nelson, 1955). From the thyroid the polythene tube passed through the sternomastoid muscle and ran a subcutaneous course to the back where it was connected to a small glass ampoule. The wound was closed in layers and the skin edges approximated with stainless steel sutures and metal clips. The animals were maintained on a post-operative penicillin and streptomycin cover. Three days later, the animals were again anaesthetized, a small incision was made in the back and a micro-injector was connected to each polythene tube going to the thyroid lobe. The wound was closed with stainless steel sutures and the injector connected to the polythene tube was left buried in a subcutaneous pocket in the back. The details of construction and use of the injector technique in biological research have been described (Rose and Nelson, 1955).

In this particular experiment the injector delivered 0·2 ml. of 1 p.c. PTU per diem to one thyroid lobe for 8 days. The PTU was buffered to pH 10 with M/10 glycine buffer. Another injector delivered the same volume of the buffered solution not containing PTU to the control thyroid lobe. Seven days after implanting the injector, the animals received by subcutaneous injection 200 microcuries of I^{131}. Twenty-four hours later the animals were sacrificed and the thyroid lobes were removed and fixed in formalin. Radioautographs and histological sections were made of the thyroid lobes around the implanted plastic tube. The radioautographs were made by floating the section on to the emulsion surface of a lantern slide; these were kept in contact in the dark for 5 days and then developed. The contiguous section was stained with haematoxylin and eosin and the height of 100 acinar cells from follicles around the tube was measured. For the thyroid lobe receiving PTU this meant that the measurement of cell height was taken from follicles with a poor uptake and organic binding of I^{131} due to the localized action of the PTU.

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[1]Working with a full-time grant from the National Health and Medical Research Council.

RESULTS.

Figs. 1a and 1b are radioautographs of the control thyroid lobe which received a continuous injection of the buffered solution not containing PTU. There is a good uptake and organic binding of I^{131} in the thyroid lobe generally and this extends to those follicles near the plastic tube. Figs. 2a and 2b are radioautographs, from the same animal, of the experimental thyroid lobe which received a continuous injection of 0·2 ml. of 1 p.c. PTU per day for 8 days. There was a poor I^{131} uptake for 2–3 mm. around the plastic tube and improving uptakes beyond this distance. The results of the radioautograph studies indicate that a localized action of PTU has been obtained in the thyroid follicles immediately adjacent to the plastic tube, without a significant effect on the control

TABLE 1.

Acinar cell heights.

Dog	Acinar cell height (μ) control thyroid lobe receiving buffer	Acinar cell height (μ) experimental thyroid lobe receiving PTU
1	1·77 ± ·16*	1·78 ± ·18
2	1·10 ± ·23	1·01 ± ·22
3	1·74 ± ·29	1·81 ± ·27

* Standard deviation.

thyroid lobe. Table 1 gives the mean acinar cell height from control and experimental thyroid lobes. The cell height from follicles which have been under the local influence of the high concentration of PTU was not higher than the control. Figs. 1c and 2c are histological sections of control and experimental lobes showing the plastic tube, connective tissue reaction and surrounding follicles. The control and experimental thyroid lobes appear to be the same. The results from these histological studies and acinar cell height measurements do not support the thesis that PTU augments the effect of TSH at the thyroid level.

DISCUSSION.

It is generally agreed that the main goitrogenic action of thiouracil is due to the increased TSH release following inhibition of thyroid hormone production. The possibility that some of the goitrogenic activity may be due to the ability of thiouracil to augment the action of TSH or reactivate TSH has been suggested. Thus TSH, which has been inactivated by exposure to implants of thyroid tissue can be reactivated by exposure to goitrogens (Rawson *et al.*, 1942). Moreover, Albert *et al.* (1947) showed that goitrogens partially restored the activity of TSH which had been treated with elemental iodine. They also showed that exposure of a pituitary extract to various goitrogens resulted in

Fig. 1A.

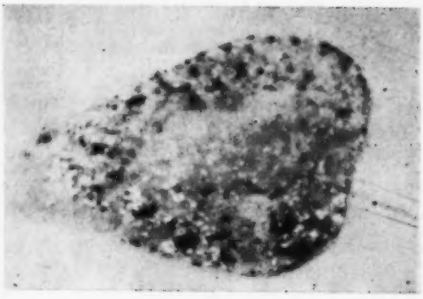

Fig. 2A.

Figs. 1A and 2A are radioautographs of the control (receiving control buffer) and experimental (receiving PTU) thyroid lobes (×8·5).

Fig. 1B.

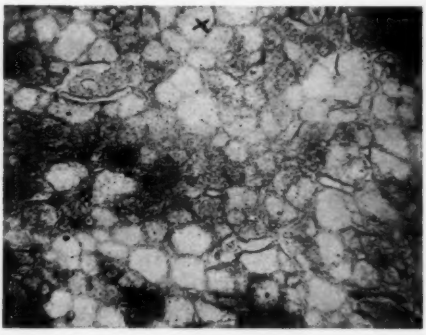

Fig. 2B.

Figs. 1B and 2B are the same radioautographs with higher magnification (×200). The tube and connective tissue reaction can be seen in Fig. 1B. The tube in the experimental thyroid lobe cannot be seen in Fig. 2B, but was present just above the point marked X.

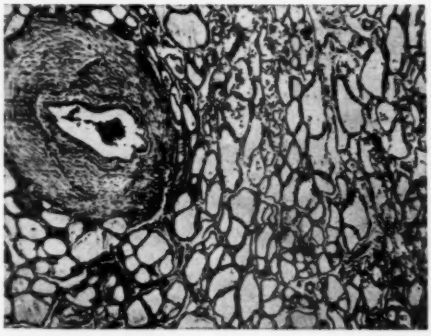

Fig. 1C.

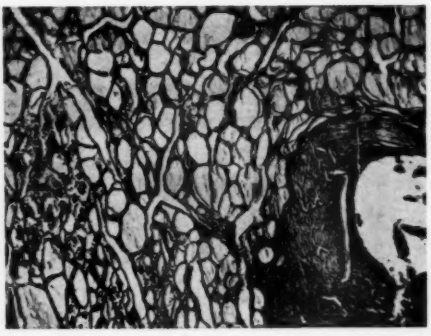

Fig. 2C.

Figs. 1C and 2C are histological sections of the control and experimental lobes showing the plastic tube, connective tissue reaction and surrounding follicles (×70).

augmentation of its TSH activity (Albert *et al.*, 1947b). Gassner *et al.* (1950) showed that PTU has an even greater augmenting effect on administered TSH than has thiouracil.

Our own results with direct continuous infusion of PTU into one thyroid lobe show that a localized inhibitory effect on uptake and organic binding of I^{131} can be achieved. Thus the volume of thyroid under the localized influence of PTU can be defined by the radioautograph technique. In this volume the thyroid follicles are not more active than the control lobe. Therefore, these results do not support the thesis that PTU augments or activates TSH *in vivo* at the thyroid level.

SUMMARY.

Using a micro-injector technique, PTU was injected by continuous infusion into one thyroid lobe of each of 3 mongrel dogs for 8 days. A localized inhibitory effect on organic binding of I^{131} was observed by radioautograph technique around the site of the PTU infusion. In this volume of thyroid tissue the follicles were not more active and the acinar cells were not higher than the control thyroid lobe. The results do not support the thesis that PTU augments the effect of TSH at the thyroid cell level.

Acknowledgment. We wish to express our appreciation to the Pathology Department of Heidelberg Repatriation Hospital for carrying out the histological and radioautograph studies.

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THE EFFECT OF SUCCINATE ON THE NUMBER OF CIRCULATING EOSINOPHILS IN MAN AND IN THE CAT

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(Accepted for publication 20th December, 1955.)

In previous publications we reported the beneficial effects of oral or intravenous sodium succinate in certain psychotic conditions (Trautner, Trethewie and Gershon, 1953; Trautner, Gershon and Duerrheim, 1954; Gershon and Trautner, 1954). The work has been extended in order to establish the safety of the therapy as well as to gain more insight into the mode of action of the drug. The effect of the drug on mineral balance, its action on renal and gastric function, on the muscular and central nervous system (including EEG studies), and on the cardiovascular system (including ECG studies) have been investigated. The observations concerning the effect of the drug on the number of circulating eosinophils are here presented separately because their significance appears to exceed that of a merely clinical control of succinate treatment. Investigations as to whether prolonged succinate treatment affects the eosinophil level of psychotic patients are in hand. The present paper, however, does not deal with the changes of eosinophil levels as observed during the course of an illness; it deals only with the changes observed within the first few hours after a single succinate injection.

METHODS.

Human subjects. Succinate for slow intravenous injection was used as a 5 p.c. sterile solution buffered with sodium hydroxide to a pH of about 6·5, 40 to 60 ml. per injection. For intravenous drip infusion, 250 to 300 ml. of a 10 p.c. solution buffered to the same pH were infused at the rate of about 2 drops per second. Both preparations were supplied by D. G. Bull Laboratories, Melbourne. In some cases succinate buffered to pH 7·4 was used; the effect on the eosinophil count was identical. The experiments on human subjects were started in the morning one hour after breakfast; for four hours thereafter no food was taken and the subject was kept at rest.

Cats. All cats used were taken from rested laboratory stock. The weight of animals ranged from 2·5 to 3·9 kg. Intact cats were left resting quietly in their cages for ½–1 hour before the first blood specimen was taken.

[1]Technical assistance for this work was aided by a grant from the National Health and Medical Research Council.

Operative procedure. The animals were induced with chloroform and ether anaesthesia, and the anaesthetic continued with ether alone. Decerebration was performed by dividing the brain stem at the level of the superior colliculi; the bleeding was controlled by vertebral artery pressure and hot saline packs. The vertebral pressure was released when clotting had occurred. The pituitary was enucleated after decerebration using cotton wool facing on the end of small-tipped curved forceps. The animals were left for ½–1 hour after operation before an eosinophil count was made; they were injected immediately after the first eosinophil count. Experimental cats were injected with 5 ml. of 5 p.c. sodium succinate solution intraperitoneally; control cats were injected either with 5 ml. saline or not injected at all. The later eosinophil counts were carried out one half hour and four hours after injection.

Eosinophil counts. Eosinophil counts were carried out by the method of Pilot (1950). In man, blood was usually obtained by finger prick, occasionally from the median cubital vein by syringe. Repeat counts on the same individual were always carried out by the same technique. Intact cats were bled from an ear vein to avoid additional stress; in the case of decerebrated and of decerebrated and hypophysectomized animals blood was removed from the femoral vein with a syringe. The counts were recorded as the number of eosinophil cells per cu.mm. of blood.

RESULTS.

Human subjects.

The subjects comprised healthy volunteers and Mental Hospital patients ranging in their condition from borderline mental defectives to temporarily "dry" alcoholics and chronic schizophrenics with exclusion of any acute or deteriorated conditions. Many of the schizophrenics were undergoing intravenous succinate treatment in their course of therapy and were used to the procedure. Most subjects were used as controls and as experimental subjects after a few weeks' interval. In others, several determinations were made at intervals of 1–2 weeks to ascertain the possible effect of apprehension or nervous stress. The basic values collected in the first count did not show a regular distribution. The observations in 70 patients collected at random were, in cells per cu.mm. blood:

below 50: 3	200–250: 9	400–450: 0
50–100: 19	250–300: 7	450–500: 1
100–150: 11	300–350: 8	500–550: 2
150–200: 10	350–400: 1	above 550: 2

The lowest count was 30 and the highest 660 eosinophil cells per cu.mm. of blood.

The initial count was taken as 100 p.c. Immediately afterwards succinate, saline or glucose was injected. The changes observed were plotted as percentage changes. The results obtained in over 70 hospital inmates are shown in Figs. 1 and 2 on a logarithmic scale to facilitate the presentation of occasional excessive percent. increases. The dotted line connects the initial 100 p.c. level with the mean values observed after 30 to 35 minutes and after 4 hours.

The controls with or without saline or 5 p.c. glucose injection showed during the observation period an insignificant increase of the mean value to the extent

of 5–7 p.c. and a moderate range of deviation from the mean. The succinate-injected patients showed within 30–40 minutes after injection a reduction of the mean of circulating eosinophils by 25 p.c., increasing to 35 p.c. within 4 hours; from then on the mean gradually returned to its initial value within 5–24 hours. Furthermore, the injection of succinate considerably increased the range of deviation; changes to values as low as 10 p.c. and higher than 300 p.c. were observed.

Figs. 1 and 2 show different symbols for the various clinical conditions. There did not appear to be any statistically significant differences in the basic counts nor in the response of dements, schizophrenics, alcoholics or the healthy

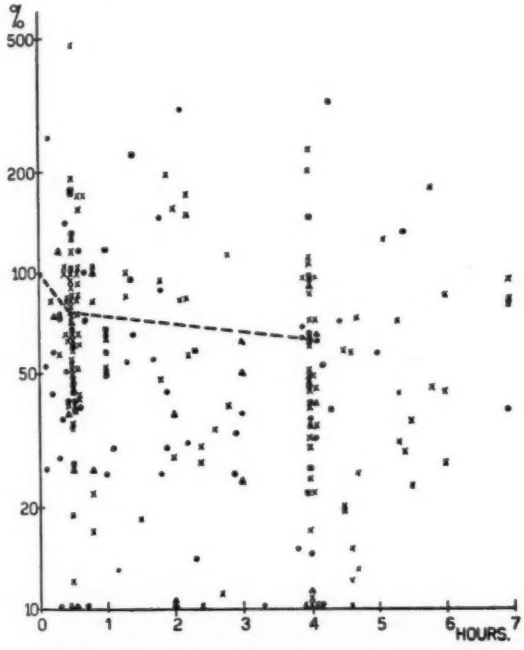

Fig. 1. Changes in the number of circulating eosinophils after injection of sodium succinate in psychiatric patients. Logarithmic presentation was chosen to facilitate the plotting of occasional extreme increases. The interrupted line connects the value of the first determination taken as 100 p.c. with the mean values determined one half-hour and four hours after the succinate injection.

Squares: Congenital mental defectives.
Triangles: Chronic alcoholics.
Dots: Schizophrenics improving under treatment.
Crosses: Untreated schizophrenics or schizophrenics who do not respond to treatment.

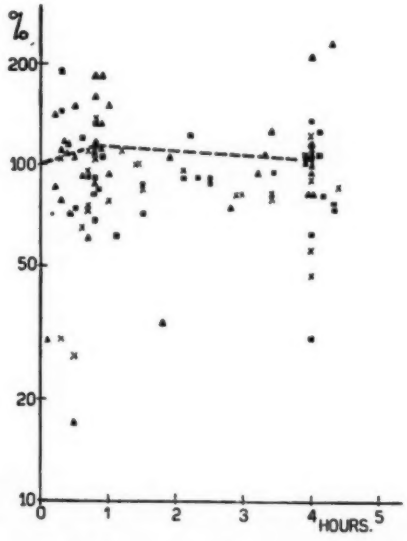

Fig. 2. Changes in the number of circulating eosinophils in psychiatric patients either without injection or after injection of saline or glucose. Markings and scale as in Fig. 1.

to succinate. Equally, attempts to subdivide the material on a scale based on prognosis or on the level of performance also failed to give convincing results. It may, however, be mentioned that two patients suffering from carcinoma of the pituitary gland and showing clinically distinct hypofunction of the pituitary-adrenal system, did not show any changes in their eosinophil level after succinate injection. Furthermore, patients who gave very erratic responses after succinate were found to be likely to give similar, if slightly less erratic responses to control injections of saline or glucose. The distribution pattern of the basic eosinophil counts does not exclude the presence of two or more as yet undefined individual "populations" and, therefore, no statistical evaluation of the results was attempted. It can only be said that, on the average, there was a distinct and significant drop in eosinophils, apparent within 30 minutes after injection of succinate and persisting over some 4–6 hours, and that the range of deviation was considerably increased in the succinate-injected subjects as compared with the controls.

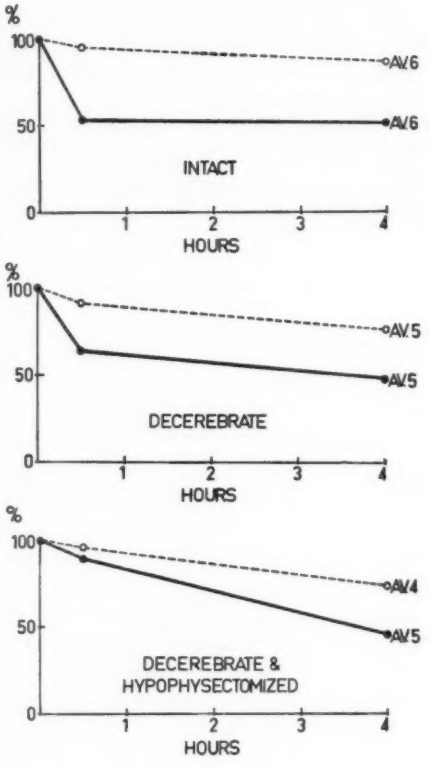

Fig. 3. Continuous line: Changes in the number of circulating eosinophils before, one half and four hours after the injection of sodium succinate in the intact, the decerebrated and the decerebrated and hypophysectomized cat. Each line represents the mean percentage change in the eosinophil count.

Interrupted line: Changes observed in control cats which were either not injected or injected with saline.

Experimental work on cats.

The mean percentage changes in the number of circulating eosinophils, as observed 30 minutes and four hours after the start of the experiment, are presented in Fig. 3 for a series of experimental and control animals.

Intact cats. Twelve cats were used. The initial eosinophil counts varied from 390 to 4050, the mean being 1132 with a standard deviation of 1082. Within five minutes of the initial count six of the animals were injected with 5 ml. of 5 p.c. aqueous sodium succinate intraperitoneally, three with 5 ml. of saline, and three did not receive

any injection. Since there was no noticeable difference between the variations in the eosinophil count of the saline-injected and uninjected control cats, these two groups were pooled. It will be seen in the top graph of Fig. 3 that the fall in the eosinophil count of the succinate-injected animals follows a pattern very similar to that observed in human subjects. If the half-hourly and the four-hour results are pooled, the difference is significant at the 0·1 p.c. level ($t=4·26$). Considering the observations in detail: After 30 minutes the eosinophil counts of the control group ranged from 72 to 114 p.c., the mean being 96 and the standard deviation 13·4. In the succinate-injected animals the eosinophil count fell within 30 minutes to 53 p.c., with a range from 37 to 79 and a standard deviation of 16·5. Compared with the controls, the difference in the half-hour values is significant by the t-test at the 0·01 p.c. level ($t=4·95$).

After 4 hours the eosinophil count of the controls had dropped to 87 p.c. with a standard deviation of 28·2 and a range from 39 to 109 p.c.; that of the succinate-injected animals had decreased to 51 p.c. with a range from 20 to 92, while the standard deviation had increased to 29·5. Owing to the increased standard deviation in both 4-hour controls and succinate-injected animals, the difference between the eosinophil counts is significant only at the 5 p.c. level ($t=2·26$), compared with 0·01 p.c. in the 30-minute values. It will thus appear that the main reduction in the number of circulating eosinophils had taken place within the first 30 minutes after succinate injection, and that later on the controls showed a further slight drop in eosinophils and a considerable increase in the standard deviation which were not observed in the control human subjects. Both these features contributed to make the difference in the 4-hour counts less significant than those in the 30-minute counts.

Decerebrated cats. Ten animals were used. The eosinophil counts one half-hour after the operation ranged from 725 to 2022 with a mean of 1458 and a standard deviation of 843. The difference from the values recorded in intact cats (mean 1158 with a standard deviation of 1082) was so highly significant (variance ratio 7·45) that it suggested that the two groups were statistically different and not comparable populations. The effect of succinate was therefore assessed not on the level of the intact cat, but on the level of the operated cat, taking the count one half-hour after operation as 100 p.c. Five animals were injected with saline and five with succinate following the same procedure used in the intact cat. It will be seen from the middle graph, Fig. 3, that the mean eosinophil changes observed in the 30-minute and 4-hour values follow the same pattern as found in the intact animals with the addition that the later decrease in the number of circulating eosinophils was more pronounced in both control and succinate-injected animals than it was in the intact animals. If the half-hourly and the four-hourly counts are pooled the difference in the eosinophil count is significant at the 4 p.c. level ($t=2·27$). Recording the observations in detail: after 30 minutes the eosinophil counts of the controls

ranged from 49 to 107 p.c., with a mean of 92 and a standard deviation of 24·4; those of the succinate-injected animals ranged from 36 to 86 p.c., with a mean of 76 and a standard deviation of 21·0. Probably, owing to the small number of animals, significance is not apparent (p=8 p.c., t=2·00). After four hours the eosinophil counts of the controls ranged from 29 to 103 p.c., with a mean of 76 and a standard deviation of 29·0, those of the succinate-injected animals ranged from 5 to 89 p.c. with a mean of 49 and a standard deviation of 33·1. Considered by themselves the four-hour values are already no longer significant (t=1·35, p>10 p.c.). It appeared thus that, as in the intact cats, the main drop in the eosinophil count took place within 30 minutes after succinate injection, that this drop was maintained in the mean values but lost progressively in significance in the later counts owing to the considerable increase of the standard deviation in both series. The pooled half- and four-hourly counts were, however, significant statistically (t=2·27, p=4 p.c.).

Decerebrated and hypophysectomized cats. Ten animals were used. The eosinophil counts one half-hour after the operation ranged from 300 to 2947 with a mean value of 1250 and a standard deviation of 843. These values were significantly different from the distribution observed in the decerebrated animals (variance ratio 4·52), but not significantly different from that observed in intact animals (variance ratio 1·65). It was not considered justifiable to assess the effect of succinate with relation to the values observed in the intact animal, and, as in the case of the merely decerebrated cat, the eosinophil count one half-hour after the operation was taken as 100 p.c. Five animals were injected with saline; one of them died shortly afterwards, leaving four control animals; five animals were injected with succinate. From the lower graph, Fig. 3, it is seen that the mean eosinophil changes follow an entirely different pattern from that found in the intact and in the merely decerebrated cats. The pronounced and statistically significant drop in the 30-minute count, which was observed in man and in the other experimental series, was absent. The four-hour counts showed an increased drop in circulating eosinophils in the succinate-injected cats, but when the 30-minute and four-hour counts were pooled, the difference was not significant, the probability being 18 p.c. (t=1·45). Considering the observations in detail: After 30 minutes the eosinophil count of the controls ranged from 90 to 106 p.c., with a mean of 96 and a standard deviation of 7·1; those of the succinate-injected animals ranged from 68 to 101 p.c., with a mean of 90 and a standard deviation of 13·5. After 4 hours the counts in the controls ranged from 43 to 97 p.c., with a mean of 74·6; the standard deviation had increased to 24·9. Those of the succinate-injected animals ranged from 33 to 63 p.c., with a mean of 51 and an almost unchanged standard deviation of 12·9. Thus it appears that there was no significant decrease in eosinophils within the first 30 minutes after succinate injection, but that in both controls and succinate-injected animals the counts decreased progressively during the time of observa-

tion. This decrease of the means was almost rectilinear: in the controls 4 p.c. within 30 minutes, reaching 26 p.c. after 4 hours. In the succinate-injected animals the rate of decrease was higher, 9 p.c. within 30 minutes, reaching 53 p.c. after four hours. Furthermore, the increase in standard deviation during the observation period which was observed in succinate-injected man, and in both controls and succinate-injected intact and merely decerebrate animals, was pronounced in the decerebrated and hypophysectomized controls, but absent in the succinate-injected decerebrated and hypophysectomized animals. The findings indicated that in the decerebrated and hypophysectomized cats there was no response to injected succinate other than the increase in the rate of progressive eosinophil drop and the maintenance of the standard deviation in the succinate-injected animals.

DISCUSSION.

The experimental findings presented show a significant reduction of the mean eosinophil count in man after intravenous administration of sodium succinate. Since the basic eosinophil counts in the subjects examined did not show a homogenous distribution, and since it was not possible to divide the human material on the basis of the eosinophil counts into clinical or diagnostic subgroups, no statistical evaluation could be attempted along these lines. It can only be said that, after succinate injection, the average eosinophil count decreased considerably during the first half-hour and slightly less during the following four hours, and that the deviation of individual counts from the mean increased considerably if compared with the observations made in the controls. The observation was confirmed in experiments with intact cats. The mode and degree of the eosinopenia caused by succinate is similar to that caused by ACTH and cortisone. Succinate therapy has been claimed to be of benefit in a variety of conditions known to respond to ACTH or cortisone: rheumatic diseases (Crain, 1951); multiple sclerosis (Aird, 1951), disseminated lupus erythematosis (Gomez Orbaneja, 1952), bronchial asthma (Frouchtman and Llovera, 1950), and others. In view of this and of the euphoric effect occasionally observed after succinate medication in the healthy, and frequently observed in depressed psychotic patients, the drug has been suggested to have "cortisone-like" action. It would be highly surprising if a straight-chain dicarboxylic acid as simple as succinic acid could replace a polypeptide or a steroid and substitute for them in their physiological or therapeutic action.

In a previous paper (Trautner, Trethewie and Gershon, 1953) we described somatic effects of succinate which could be interpreted as analeptic effects. Following prolonged drip infusion of a 10 p.c. solution, excessive over-breathing and increases of blood pressure from 140 to over 200 systolic were noted; in a few cases short excessive diuresis occurred to the extent of 2–3 litres of urine within a few hours. Barrett's (1949) observations on barbiturate coma and our

observations in various comatose conditions (prolonged insulin coma, prolonged unconsciousness after concussion or brain operations, toxic coma and similar conditions) demonstrated temporary lightening of deep coma with resumption of gastric and renal functions, and awakening from light coma. Schueler (1948) reported that, out of a number of glucose metabolites tried, succinate alone dramatically abolished experimental mescaline hallucinations; we were able to confirm this observation. All these effects can be assumed to be regulated from centres situated between the medulla and the hypothalamus or the reticular formation of the brain stem. It was, therefore, considered that succinate might exercise a generalized, diffuse stimulation in this region, which in its course results in a stimulation of the pituitary-adrenocortical system. Experiments were therefore performed to establish whether circumscribed excision would abolish the succinate effect on the number of circulating eosinophils.

As a first experimental approach, decerebration above the superior colliculi and decerebration plus hypophysectomy were selected. The number of experiments was limited by a shortage of experimental animals. The observations made about the effect of the operation itself on the eosinophil level of the animals were not the primary aim of the present investigation, and, in view of the limited amount of experiments, they can be presented as suggestive only. It appeared that decerebration decreased the standard deviation compared with the values recorded in intact cats. It must therefore be considered that the eosinophil levels of the decerebrated cat present a statistically different "population," and, that the severing of the connection of the pituitary gland from the hypothalamus interrupted the functioning of some sensitive mechanism responsible for the higher degree of variance and the lower mean observed in the intact cat. Hypophysectomy plus decerebration shifted the mean eosinophil count back towards the value observed in the intact cat and almost restored the earlier high standard deviation. It does not seem plausible to consider that —with regard to the eosinophil levels—hypophysectomy counteracts the effect of decerebration. It would rather seem that the removal of the pituitary gland destroyed a governing mechanism exercising a restricting influence, with the result that the eosinophil values either respond erratically to whatever somatic factors may affect them or that they remain as scattered as they were before the operation. It would appear that the mechanism of the regulation of the number of circulating eosinophils differs in the intact, the decerebrate and the decerebrated and hypophysectomized cat and that all that can be observed is the effect of succinate on the eosinophil level as existing after the operation.

The removal of the cerebrum above the superior colliculi caused by itself a slow decrease in the eosinophil levels within the period of observation. The degree of this reduction was small, the mean values being 8 p.c. within 30 minutes and 24 p.c. within 4 hours, but the observation was constant. This effect may be due to the stress of the anaesthetic and the operation, to the rigors induced

or to undefined somatic factors. The injection of succinate caused a further reduction in circulating eosinophils in the decerebrated cat. This additional reduction was statistically significant at the 5 p.c. level. It appeared thus that the centres situated above the superior colliculi, vegetative or otherwise, and the intactness of the normal circulation through the pituitary stalk were not necessarily concerned in the response of the eosinophils to injected succinate. (This observation refers, of course, only to the response to succinate and is not intended to imply that these centres or connections take no part in the regulation of the number of circulating eosinophils in the intact animal or by other agents.) The standard deviation in the half-hourly counts was higher than in the intact cats, and it increased further during the four hours of observation, slightly more in the succinate-injected animals than in the controls.

When, in addition to decerebration, the pituitary gland was also removed, an entirely different picture presented itself. The steep decrease in circulating eosinophils observed in the intact and merely decerebrated cat within the first half-hour after succinate injection was completely absent. Instead, a slow, almost rectilinear decrease persisted in both series throughout the period of observation. The character of this decrease was similar to that shown in the decerebrated controls. The decerebrated and hypophysectomized controls showed about the same reduction (4 p.c. within 30 minutes and 26 p.c. within 4 hours), as the merely decerebrated controls (7·3 p.c. in 30 minutes and 24 p.c. within four hours). In two experiments on the adrenalectomized cat, while saline produced no effect on the eosinophil level, a reduction of 30 p.c. occurred in the case of succinate injection.

It appeared thus that the additional operation of hypophysectomy did not contribute essentially to the already existent trend towards a decrease in the number of circulating eosinophils. The succinate-injected animals showed a fall of 10 p.c. within 30 minutes and one of 53 p.c. within four hours. No indications could be found why, in the absence of the initial succinate effect, the succinate-injected animals showed a steeper decrease in eosinophils than the controls, nor why the increase in the standard deviation shown by both series of intact and merely decerebrated cats, though still present in the decerebrate and hypophysectomized controls, was absent in the succinate-injected animals of this series.

Salicylates depress the eosinophil count in a similar way. Trethewie (1952, 1954) observed that they exercise an additional specific cellular effect which cannot be accounted for by the release of ACTH. Possibly succinate also exercises some specific somatic effect and this may explain the higher rate of the decrease in circulating eosinophils in the succinate-injected animals of this series as compared with that of the controls. Our observations show that the presence of the pituitary gland is largely essential for the characteristic early effect of succinate on the eosinophil count, and that the presence of the cerebrum above the superior colliculi is less essential.

The observation concerning this rôle of the pituitary gland was disturbing in so far as the main blood supply of the gland is derived from cerebral vessels damaged during decerebration. Even if succinate, which encounters little or no blood-brain barrier, could be assumed to diffuse into the isolated pituitary gland, it was difficult to understand how the pituitary response, ACTH or any other, could have been transmitted. Green (1951) described an additional blood supply for the pituitary gland of the rabbit. No similar observations about the cat appear to have been published. We noted, however, when, following decerebration, the tent of the dura covering the pituitary was penetrated, very considerable bleeding occurred at the posterior margin of the sella turcica. Further bleeding was noted while the remains of the gland were enucleated. Furthermore, when after intraaortic injection of Indian ink into the decerebrated cat, the pituitary gland was examined histologically, some ink particles were found to have penetrated into the gland substance, though mainly peripherally. These observations suggest that in the cat there is also some supplementary vascular connection of the pituitary gland with the peripheral circulation, and that its extent is sufficient to allow not only the access of succinate but presumably also the transmission of the pituitary response.

A reduction of circulating eosinophils of the same character as that shown by succinate is observed after the injection of adrenaline, ephedrine, histamine and related amines, and, furthermore, after that of chemically quite unrelated compounds like salicylic or *p*-aminosalicylic acids. Favez *et al.* (1954) note that oral PAS has comparatively little effect on the eosinophil count and considered that "the higher blood concentration of intravenously administered PAS acts as a stressing agent and induces the release of ACTH from the pituitary." Although the release of corticoids and of ACTH in blood or urine was not confirmed by Smith *et al.* (1954), and by Bayliss and Steinbeck (1954) using salicylate, it remains to be considered whether succinate may act as a stressing agent. Observations on ourselves and on patients suggested that pronounced respiratory and circulatory stimulation was found only after the intravenous injection of succinate in amounts considerably higher than those required for the eosinophil response and that even in the presence of respiratory or circulatory stimulation there was no indication of tension, irritability or other symptoms of stress. Our healthy subjects did not feel any difference in their condition before and after the intravenous injection. In the experimental animals the drug was administered intraperitoneally, a mode of application which would not cause an acute increase in the succinate concentration in the circulating blood. Intact cats, dogs, guinea-pigs and rats injected intraperitoneally or intravenously with doses of succinate comparable with, or higher than those used in the present work, never showed any signs of distress and were, as soon as they were put back into their cages, indistinguishable in their behaviour from the controls which had not received any injection. Furthermore, the function of the

pituitary gland is usually accepted as being regulated by neuro-humoral or humoral transmitters originating from the hypothalamus. It may be questionable to what extent a somatic stress caused by intraperitoneal injection can reach the pituitary gland in the decerebrate cat. We do not question that a drop in the circulating eosinophils forms part of the systemic stress response, but we are not convinced that succinate acts as a stressing agent. Our observations show only that even in the absence of the cerebrum above the superior colliculi the pituitary gland responds to succinate with respect to the eosinophil count in the same way as the intact animal responds to the principal stress agents or to the physiological amines released from the adrenals. It may tentatively be considered whether succinate substitutes in the decerebrate animal for the normal humoral hypothalamic transmitter.

Cauwenberge (1951) reported that the biochemical and haematological signs of adrenocortical hyperactivity observed after large doses of salicylates are no longer observed in the hypophysectomized rats. Two of our patients suffering from underfunction of the pituitary gland did not respond to succinate with any eosinophil changes. Sayers (1950) stated that large doses of adrenalin can induce eosinopenia even in the absence of the adrenal cortex, and Elmadjin (1954) found that in adrenalectomized patients maintained with adrenocortical extracts the eosinophil response to injected adrenalin was unaltered. We have so far few observations with regard to the rôle of the adrenals in the mechanism of the succinate-caused eosinophil response. It would be of interest to establish to what extent the various agents producing eosinopenia in the intact animal depend on the intactness of the whole stress-response chain or to what extent some agents may act directly on one single link like the pituitary gland, hypothalamus, or adrenals, and so cause the eosinophil effect even if other links are destroyed. It is intended to continue the investigation on these lines.

SUMMARY.

Intravenously-administered sodium succinate caused in healthy man, as well as in patients suffering from various psychotic conditions, a rapid decrease in the number of circulating eosinophils during the first half-hour after injection. This decrease was maintained for about four hours. The response was of the same character as that seen after intravenous administration of adrenalin, ACTH and related drugs.

The same response was seen in the intact cat after intraperitoneal administration of the drug.

The experimental results suggested that the eosinophil levels in the intact, the decerebrated and the decerebrated and hypophysectomized cat presented statistically different and not comparable populations. The effect of succinate was therefore assessed in relation to the eosinophil level present one half-hour

after the end of the operation. It was found that the response was not significantly affected by decerebration above the superior colliculi; but it was abolished when the pituitary gland was enucleated.

The mechanism of this response is briefly discussed.

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THE EFFECT OF SEASON AND MODE OF TRANSMISSION ON THE SEVERITY OF MYXOMATOSIS DUE TO AN ATTENUATED STRAIN OF THE VIRUS

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Infection of rabbits with the higher virulent standard laboratory strain of myxoma virus is so overwhelming that possible effects of minor degrees of host resistance, of seasonal conditions, or of mode of transmission cannot be manifested. However, during studies of the attentuated Uriarra strain of myxoma virus (Mykytowycz, 1953), considerable differences occurred in the severity of clinical symptoms and in the mortality rate. Some of these differences were found to be associated with the mode of transmission of the disease, and climatic conditions were also found to exert an effect on the mortality rate.

Materials and Methods.

Virus.

The Uriarra strain (Mykytowycz, 1953) was used for most experiments, but some experiments were also carried out with the KM 13 strain (Myers, Marshall and Fenner, 1954). The direct contact line strain of Uriarra from the original rabbit recovered from the field (UC), and the strain obtained from the third rabbit of this series (U3) have been differentiated in the text, although their behaviour in rabbits is identical.

Rabbits.

Adult wild rabbits were used; they were caught in various localities in the Australian Capital Territory. As myxomatosis occurred in these areas and immune rabbits were not uncommon, the blood of all rabbits used was first tested for antibodies. Complement fixation and neutralization tests were performed following the techniques described by Fenner, Marshall and Woodroofe (1953). Only rabbits showing no serological evidence of recovery from myxomatosis were used.

Mosquito transmission.

Female *Aedes aegypti* (L.) were kindly supplied by Dr. M. F. Day, of the Division of Entomology, C.S.I.R.O. They were bred and kept in an insectary with a constant temperature of approximately 27° C., and relative humidity of 60–80 p.c. The technique used for mosquito transmission experiments was described by Fenner, Day and Woodroofe (1952). The individual mosquitoes were kept in glass vials, the ends of which were closed by small pieces of mosquito netting. Each was allowed to probe the skin lesion on a donor rabbit, and was then allowed to engorge on a marked skin site on the back of a healthy rabbit.

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Cage contact.

Contact transmission of the disease was effected by keeping a sick and a healthy rabbit together in a wire cage, measuring 18×12×10 inches, during the whole course of the disease. In some cases where fighting endangered the health or life of one of the animals, the contact time was shortened to 16–24 hours, which was found to be sufficient for transmission of the disease. When it was necessary to infect a larger number of rabbits from one donor, five or six healthy animals and one sick one were placed in a large wire cage, measuring 24×24×11 inches. The length of the incubation period and the survival time of rabbits infected by contact have been calculated from the time of first contact of a healthy with a sick rabbit.

Inoculations.

Rabbits were inoculated intradermally with 0·1 ml. of inoculum containing approximately 10 ID_{50} of virus. Virus material was harvested from affected skin tissue, which was ground with alundum, and suspended in borate-buffered saline, containing 0·5 p.c. gelatine. It was titrated on the chorioallantoic membrane of the developing chick embryo (Lush, 1937) and the dosage for rabbit inoculation calculated as described by Fenner and Woodroofe (1953).

Special precautions were taken to avoid virus being deposited in the skin tissue during intramuscular inoculation. The standard amount of inoculum (0·1 ml.) was drawn into the syringe, allowing an air space both above and below it. The needle was changed before the injection was made. The absence of primary skin lesions at the sites of inoculation indicated that intramuscular injections, without contamination of the skin, had been achieved.

In carrying out intranasal and intraocular inoculations, 0·05 ml. inoculum containing 5–10 ID_{50} of virus was dropped into each nostril and conjunctival sac respectively by means of calibrated Pasteur pipettes.

"Pure clone" virus preparations were obtained by two methods. In the first a dilution of virus which produced very few pocks was inoculated on the chorioallantoic membrane. A single pock was harvested three days later from a membrane on which only one pock occurred, passed once intradermally in a rabbit, and used as the "pure clone" preparation. The second method depended upon the harvesting of the skin lesion produced at one out of eight or more sites on the rabbit's back by a limiting dilution of the virus preparation. Fenner and McIntyre (1956) have shown that by both of these methods it is possible to obtain a virus suspension derived from a single infectious dose of myxoma virus.

EXPERIMENTAL RESULTS.

Comparison of infections initiated by contact and mosquito bite.

Using as the initial donor a wild rabbit infected naturally in the field with the Uriarra strain, a series of 36 successive susceptible wild rabbits was infected over a period of nine months by means of contact in a cage with a sick rabbit. In all instances, when a single susceptible rabbit was put in cage contact, the symptoms of generalization appeared within 6 to 9 days. When several healthy rabbits were in contact with the sick animal the incubation period often varied from one animal to another. Only five out of 36 animals (14 p.c.) died in the course of the series of contact infections. The mean survival time of the fatal cases was 26 days, ranging from 20 to 39 days. This may be compared with the 100 p.c. mortality rate and mean survival time of 11·1 days found with the standard laboratory strain (Fenner and Marshall, in preparation).

As the initial contact experiments with the UC strain were associated with a

high recovery rate, it was surprising to find during experiments upon the transmissibility of the Uriarra strain by *Aedes aegypti* (Fenner, Day and Woodroofe, in preparation), that 17 out of 18 infected animals died within 13 to 30 days. The donor for this mosquito transmission series was a rabbit which had been inoculated with approximately 10^4 ID_{50} of virus harvested from the third animal of the contact series (i.e., strain U3).

To see whether the observed greater severity of the disease was due to mosquito transmission, a series of alternate contact and mosquito transmissions was carried out. From group (A) of mosquito-infected rabbits, a donor was used to infect by contact rabbits of series (B). In succession, a rabbit of this group was used as a donor for a second group (C) of mosquito-bite transmissions. Again rabbits from group (C) were used as donors for a second contact series (D). Following this pattern, the third group of mosquito-bite transmission (E) and a third series of contact transmission (F) were established. The results, which are shown in Table 1, clearly demonstrate a higher rate of mortality among rabbits infected by mosquito bite. Twenty-nine out of a total of 35 rabbits infected by mosquito bite died, whereas there were only 14 fatal cases out of 65 animals infected by contact.

Mosquito transmisison experiments were also carried out with UC strain. The donor rabbit was one of the direct Uriarra contact series. It showed the symptoms of a mild type of infection, with numerous skin lesions of a flat, sharply defined appearance, and dark purple in colour. Two out of 10 rabbits infected by mosquito bite died. Among the 10 rabbits infected by contact at this same period of the year not one died, but the difference between these mortality rates is not statistically significant.

The clinical symptoms of the disease showed even more clearly than the mortality rate that, with the Uriarra strain, the method of transmission influenced the grades of severity.

Three different grades of severity were noted during observations upon this strain.

Mild: An extremely mild to mild course of the disease was manifested by symptoms which often made positive diagnosis difficult. There were small, hard, red swellings up to $\frac{1}{2}$ cm. in diameter scattered all over the body, but especially on the forequarters. They were sometimes very sparse. General swelling of the eyelids did not occur, although small localized swellings were sometimes found on one or both eyelids. There was no discharge from the eyes or nose. The perineal region was either normal, or showed small discrete asymmetrical swellings. Skin lesions were often more easily observed during the regressive stage of the disease, when crusts formed on affected parts of the skin and, after peeling off, left bare patches. The general condition of rabbits with this type of disease was not adversely affected.

TABLE 1.

Comparison of the mortality rates of rabbits infected with the UC or U3 strains of myxoma virus by contact infection, or by mosquito bite.

Mosquito bite transmission				Contact transmission			
Series	Initiated by rabbit from	Number of infected rabbits	p.c. mortality	Series	Initiated by rabbit from	Number of infected rabbits	p.c. mortality
U3 Strain							
A	inoculated U3	17	100	B	series A	12	50
C	series B	7	100	D	series C	20	20
E	series D	11	45·4	F	series E	33	12·1
	Total	35	82·8		Total	65	21·5
UC Strain							
	Uriarra contact	10	20		caught in the field	36	13·8

TABLE 2.

The severity of myxomatosis due to contact infection or mosquito bite infection of wild rabbits with the U3 and UC strains of virus.

Contact					Mosquito bite					Intradermal inoculations with "pure clone" material				
Series	Mild	Moderate	Severe	Total infected	Series	Mild	Moderate	Severe	Total infected	Series	Mild	Moderate	Severe	Total infected
U3 Strain														
B	1	6	5	12	A	0	1	16	17	166A	0	1	4	5
D	8	6	6	20	C	0	0	7	7	166B	0	0	5	5
F	10	12	11	33	E	0	4	7	11	420	0	1	4	5
										399	0	2	3	5
Totals	19	24	22	65		0	5	30	35		0	4	16	20
Percentages	29·2	36·9	33·8			0	14·3	85·7			0	20	80	
UC Strain														
	3	21	12	36		0	2	8	10		0	0	6	6
Percentages	8·3	58·3	33·3			0	20	80			0	0	100	

Moderate: A moderately severe course of the disease was characterized by numerous hard, clearly-defined swellings up to 1 cm. diameter. These were most numerous on the forequarters. The eyelids were always swollen but rarely to the extent that the eyes were closed. Usually these swellings on the eyelids took the form of two or three large clearly demarcated tumours, and similar tumours occurred on the nose and face as well. The eye and nasal discharges were slight. The perineal region was always red and swollen. As in the mild cases, the general condition of the rabbit remained fairly good.

Severe: The symptoms of severe type resembled those produced by the highly virulent standard laboratory strain, except that the disease progressed much more slowly. Swellings of the head, eyelids, perineal region, and at the site of the primary infection in the case of rabbits infected by mosquito bites were very extensive and severe, with edges which merged into the normal skin. Tumours with clearly demarcated edges appeared three weeks after generalization in cases where the rabbits eventually recovered. The eyes always became closed. Nasal discharge was a fairly constant symptom in severe cases; it blocked the nostrils and caused difficulty in breathing. All animals became very thin and susceptible to secondary infections.

If the cases are analyzed according to these criteria of severity, it is clear that mild cases were much more common among the rabbits infected by contact than among those infected by mosquito bite or by intradermal inoculation. The symptomatology and severity after intradermal inoculation closely resembled those found in rabbits infected by mosquito bites.

A comparison of intradermal and intramuscular inoculation.

When it became evident that different methods of transmission were associated with different degrees of severity of the disease, it was decided to test the effect of intramuscular inoculation.

Two groups each of six wild rabbits were inoculated with 10 ID_{50} of a "pure clone" UC strain, one intradermally and the other intramuscularly. Each rabbit of the intradermal group served as a donor to a normal kitten, kept in contact all the time in the same cage. The results shown in Table 3 were clear cut. All six rabbits of the intradermal group manifested severe symptoms, three of them dying. The symptoms in the rabbits inoculated intramuscularly varied. One animal did not show any symptoms. Complement fixation test revealed antibodies in the blood, and this rabbit may have recovered from an earlier natural infection. The course of the disease in the other rabbits was very mild in one animal, moderately severe in three, and severe in one. All recovered.

All rabbits in contact with the group inoculated intradermally contracted the disease, but none died. Two cases were mild and four moderately severe. After approximately three weeks, when the surviving intradermally-inoculated rabbits were still showing severe symptoms, the condition of the cases infected by contact was so good that they could be used for other experimental purposes.

TABLE 3.

Comparison of the infection of rabbits by various routes with a pure clone derivative of the UC strain of myxoma virus.

Dosage for inoculation: 10 ID_{50}

Mode of inoculation	Proportion infected	Survival times	Severity		
			Mild	Moderate	Severe
Intradermal	6/6	20, 28, 45, S, S, S	0	0	6
Contact infection from intradermal inoculations	6/6	S, S, S, S, S, S	2	4	0
Intramuscular	5/6*	S, S, S, S, S	1	3	1
Intranasal	5/19	16, 20, S, S, S	1	0	4
Conjunctival	7/25	22, 25, S, S, S, S, S	4	1	2

* One animal failed to show symptoms.

Infection by conjunctival and intranasal inoculations.

As intramuscular transmission is most unlikely to occur naturally, ways of transmission which could occur under field conditions were simulated. It has been shown by various investigators (Martin, 1936; Bull and Mules, 1944; Houlihan and Lawson, 1945) that myxomatosis can be transmitted via the respiratory route and intraocularly. Only a small proportion of rabbits was infected by the intranasal or conjunctival instillation of 10 ID_{50} of virus, five out of 19 inoculated intranasally, and seven out of 25 inoculated in the conjunctival sac. In both groups the severity of the disease ranged from fatal to very mild, and there was no significant difference in the severity as between the two groups. If the twelve rabbits inoculated by these two routes are compared with rabbits infected by mosquito bite or intradermal inoculation (Tables 2 and 3), it appears that the range of severity is intermediate between that found after contact and intradermal or mosquito-bite infection.

Variation in mortality rate with season.

Analyzing the data obtained in experiments with both the UC and U3 substrains, it was found that the mortality rate varied considerably at different times of the year. The U3 contact series was established in July, 1953. The mortality rate started to rise in August, reached its peak in September, and declined to zero in January-May (1954) period. It started to rise again in June, 1954, and continued to rise until observations ceased.

Observations on the UC strain lasted for a shorter period, from February, 1953, till November, 1953, but the mortality curve followed the same pattern. There were no deaths during the February-April period, after which the mortality curve fluctuated, reaching its peak in September, and then declined steeply.

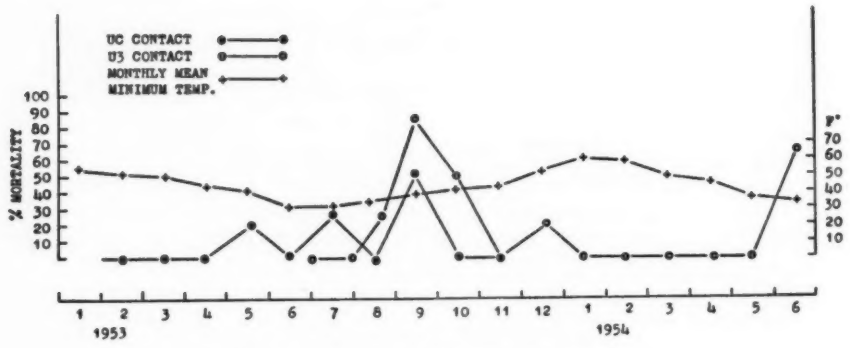

Fig. 1. THE SEASONAL FLUCTUATION OF MORTALITY RATE IN RABBITS INFECTED WITH "URIARRA" STRAINS OF MYXOMA VIRUS.

With both strains the period of highest mortality was from May-September, the winter months in Australia. The mean minimum temperatures in Canberra, where these observations were carried out, varied from 31–41° F., and the rabbits were kept in an unheated animal house. The seasonal effect was also evident in the mosquito-infected groups, although not so clearly owing to the lack of continuity of the series. Seasonal variation in symptomatology ran more or less in parallel with the mortality rates. The distinction between the mortality rates associated with contact and mosquito infection was most pronounced during the cold season.

Examination of pure clones derived from single pocks.

Jacotot, Vallée and Virat (1955) described the results of infection of laboratory rabbits by contact and intradermal inoculations using the U3 strain of virus. They observed an overall mortality rate of 67·7 p.c. and noted that severity of cases varied greatly. From this they concluded that U3 strain was a mixture of highly virulent and an attenuated strain of virus. Experiments conducted with "pure clone" lines of U3 virus do not support this view.

Four "pure clone" preparations of the U3 strain were used. Two originated from single pocks on different chorioallantoic membranes, one of these (420) being supplied by Mr. I. D. Marshall. Two others (166A and 166B) were derived by the intradermal inoculation of rabbits. The lesions were reaped on the fourth day, before contamination could occur due to spread of virus from other primary lesions in the same animal (Fenner and Woodroofe, 1953). These preparations were supplied by Professor Fenner.

After titration of each of these preparations, groups of five wild rabbits were inoculated intradermally with doses of 10 ID_{50}.

TABLE 4.

The results of the intradermal inoculations of rabbits with doses of 10 ID_{50} of five "single pock" isolates of the U3 strain of virus.

Isolate number	Source	Survival times (days)	Mean survival time
166A	Rabbit titration	23, 24, 24, S, S	38·2
166B	Rabbit titration	12, 21, 29, 31, S	30·6
420	Single pock on chorioallantoic	15, 28, 34, 48, S	37·0
399	Single pock on chorioallantoic	18, 23, 35, S, S	39·2

S = survivor: allotted 60 days survival time in calculating the mean survival time.

In each group of five animals there were some severe fatal infections, some prolonged, but eventually fatal infections, and milder cases which progressed to recovery.

Relationship between the stage of disease in the donor rabbit and the severity of infection in the recipient rabbit.

Hudson and Mansi (1955), working with attenuated English strains derived from rabbits in the Sherwood Forest area in Nottinghamshire, found that the mortality rate of inoculated rabbits depended on the stage of the disease in the donor rabbit. Infections transferred 3–6 days after inoculation were associated with a mortality rate of 89 p.c., and the rate diminished until virus transferred between 22 and 49 days after inoculation killed only 14 p.c. of the inoculated rabbits (Table 5). In the experiments reported in this paper, the material for intradermal inoculation was always obtained early in the disease. Mosquito

TABLE 5.

Comparison of mortality rates in rabbits infected by contact with Uriarra strains of virus and grouped according to the duration of the disease of the donor.

Series	Days after infection at which sick animals served as donors to contact-rabbits							
	3-6		7-9		10-21		22-49	
	Infected	Fatal	Infected	Fatal	Infected	Fatal	Infected	Fatal
UC contact	—	—	3	0	19	2	13	3
U3 B	—	—	4	2	6	2	2	2
U3 D	—	—	4	0	10	1	6	3
U3 F	—	—	6	0	22	4	5	0
Totals	—	—	17	2	38	7	13	5
Percentage mortality	—	—		12		16		31
Hudson and Mansi (1955) percentage mortality	18	89	8	75	19	53	7	14

transfers were made between the fifth and eighteenth days, usually on the seventh or eighth day. Among the rabbits infected by contact there were animals which contracted infection from donor rabbits at all stages of the disease. The available data are presented in Table 5, along with those of Hudson and Mansi. It is clear that the trend of the severity of contact infections with the Uriarra strain was the reverse of that encountered by Hudson and Mansi, although the differences in mortality rates were not significant. The failure to contract infection of any rabbits exposed by contact between the third and sixth days of the disease in the donor rabbit is not surprising, since at this stage the virus is not shed, nor are ectoparasites likely to have become infected.

Comparison of contact and mosquito infections with the KM 13 strain.

Tests comparing mosquito and contact modes of transmission were carried out with a slightly attenuated Australian field strain KM 13, in order to ascertain if the results obtained with Uriarra strains were of general application. Intradermally inoculated rabbits were used as donors for a mosquito-infected group of 15 rabbits, and a contact series of 14 rabbits. All rabbits from both the mosquito-infected group, and the contact series died. The duration of the disease in days is shown in Table 6. All animals exhibited severe symptoms and there was no significant difference between the two groups.

TABLE 6.

Comparison of mosquito bite and contact infections of rabbits with KM 13 strain of virus.

Mode of inoculation	Survival times	Mean survival time
Mosquito bite	12, 12, 12, 13, 13, 13, 13, 13, 13, 14, 14, 14, 14, 22, 26	13·2
Contact	12, 13, 13, 14, 14, 14, 14, 14, 15, 15, 16, 17, 18, 18	14·8

DISCUSSION.

The response of *Oryctolagus cuniculus* to infection with the highly virulent standard laboratory strain of myxoma virus, and to the virulent Lausanne strain, used to initiate the disease in Europe, is extremely uniform. In the laboratory cases are almost invariably fatal, and the progression of symptoms is similar in almost all animals infected (Fenner and Marshall, 1956). The response of rabbits to infection with the attenuated Uriarra strain, on the other hand, varies considerably, some cases progressing relatively rapidly to death (though never as rapidly as in infections with the highly virulent strains), while other cases are very mild and are characterized by small localized tumours, without general oedema or noticeable interference with the general health of the infected animal.

Jacotot and his collaborators (1955) observed a similar diversity in the clinical picture in rabbits infected with the Uriarra strain, and they suggested

that the strain was a mixture of highly virulent virus and a rather attenuated form. The investigations with "pure clone" isolates do not support this interpretation. It appears more probable that the milder nature of the disease which follows infection with the Uriarra strain allows the expression of differences in the innate resistance of different rabbits which are obscured by the overwhelming virulence of the standard laboratory strain.

There is suggestive evidence that certain other field strains may in fact be mixtures of infections. The observations of Hudson and Mansi (1955) may have some such explanation, and Fenner and Marshall (1955) suspected on other grounds that the Nottinghamshire strain of Hudson and Mansi may be mixed. Further work is necessary to elucidate this situation. It is noteworthy that the slight differences which occurred in the mortality rates of rabbits infected by contact with donor rabbits at different stages of the disease showed a trend the reverse of that reported by Hudson and Mansi.

The differences in the mortality rate and the severity of the disease in rabbits infected by contact, or by mosquito bite, to take the two natural modes of infection, are unequivocal and reproducible, although they were not observed with the slightly attenuated strain KM 13. No explanation can be advanced for these differences.

The greater severity of infections in the cold weather observed with the Uriarra strain accords with the experience of Sobey (unpublished) with the KM 13 strain. Summer temperatures in Canberra are not high enough for the temperature effect described by Parker and Thompson (1942) to operate, and it appears that exposure to cold weather in unheated animal rooms decreases the resistance of the animals to myxomatosis.

The epizootic spread of myxomatosis in Australia is undoubtedly due to the activity of winged insect vectors. The significance of contact infection is more difficult to evaluate, but it may play a part in the over-wintering of the disease at a very low level of intensity, for cases infected by contact may remain mobile throughout the infection, and can act as sources of virus for contact infection of other rabbits up to two or three months after contracting the disease.

The rôle of secondary infections in outbreaks of myxomatosis is difficult to assess. In the catastrophic epizooties due to highly virulent strains the activity of the virus overshadows all other factors. With the more attenuated strains, however, such secondary infections, which may be provoked into activity by the virus disease, may play an important part in increasing the mortality rate in wild rabbits under field conditions. The adverse effect of low temperatures may also increase the mortality rate in winter outbreaks of myxomatosis caused by attenuated strains of the virus.

In addition to the three grades of severity mentioned earlier, a fourth one —prematurely lethal—occurred occasionally. Some animals infected with the virus died in the early stages of the disease (6–13 days), before the development of normal symptoms of myxomatosis. Although virus capable of producing

symptoms characteristic of the Uriarra strain could be recovered from the carcasses, these animals have not been included in the analyzed data as in none of them was myxomatosis the primary cause of death. Post-mortem examination revealed that in all these cases the disease was augmented by secondary factors, and the pathological changes were not those typical of myxomatosis. Bacteriological and parasitological examinations showed the occurrence of coccidia, *Bact. coli*, haemolytic streptococci, diplococci, and *Fasciola hepatica*. In other cases the stomach worm, *Graphidium strigosum*, caused perforation of the stomach wall and peritonitis. Prematurely fatal cases occurred among mosquito-infected rabbits (9 out of 54) as well as amongst contact infections (5 out of 106) and were more prevalent during the cold season.

SUMMARY.

The severity of the disease, and the mortality rates, due to infection of wild rabbits with the attenuated Uriarra strain of myxoma virus, were greatly influenced by the mode of infection. Mosquito-bite infections and intradermal inoculation caused much more severe disease and higher mortality than did contact infections.

The severity of the disease was also affected by temperature, cases being more severe and associated with a higher mortality rate in winter, compared with warmer times of the year.

Study of "pure clone" isolates of the Uriarra strain gave no indication that this strain was a mixture of a highly virulent and a greatly attenuated form of the virus.

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EFFECTS OF PANCREATECTOMY IN THE SHEEP

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The diabetic syndrome which follows complete removal of the pancreas from non-ruminant animals differs in some respects from that which ensues after the injection of a diabetogenic dose of alloxan (Thorogood and Zimmermann, 1945).

In ruminants, however, the diabetic syndrome has been reported to be relatively mild (Lukens, 1948), whatever the means employed to induce it. Observations on goats made diabetic by pancreatectomy suggest that only a mild glycosuria or ketonuria supervenes (Lukens, 1938; Greeley, 1947), and Cook, Dye and McCandless (1949) have observed only a moderate diabetes in depancreatized calves. Blomback *et al.* (1953) kept a depancreatized sheep alive for 2 months without treatment with insulin.

In contrast to these reports, Jarrett (1946), and McCandless, Woodward and Dye (1948) observed severe glycosuria and ketonuria in alloxan diabetic ewes, and Saviano and De Franciscis (1946) have found a moderately severe alloxan diabetes in goats. A serious ketosis prevails in Merino rams and wethers when they are rendered diabetic by alloxan injection (Jarrett and Filsell, unpublished data). Young male calves, however, have been reported to be refractory to alloxan (McCandless and Dye, 1949).

In the studies reported here the apparent variations in the severity of the diabetic state in ruminants have been further investigated in depancreatized sheep and young lambs.

In the sheep, the pancreas is so diffusely distributed and so intimately adherent to the blood vessels that complete removal is an especially difficult task. For the studies reported here a surgical procedure was developed for the removal of the pancreas.

OPERATIVE PROCEDURE.

Under nembutal anaesthesia, the skin over the operation site on the right side was prepared and an incision made along a line approximately 3 cm. from the costal arch extending from the xiphoid to the transverse process of the lumbar vertebrae.

The pancreas was approached from the right lateral aspect and the pancreatic duct located and severed between ligatures placed as near as possible to its entry into the bile duct.

The pancreas was freed along the course of the bile duct and small intestine, meticulous care being taken to remove small portions of the pancreas closely adherent to the medial surface of these structures. The peritoneum was cut along the posterior and dorsal borders of the pancreas about 1 cm. out from its edge. The pancreas was reflected from the underlying structures, beginning from the posterior border and proceeding until a vein leaving the pancreas to join the gastro-duodenal vein was displayed on the medial surface; it was then reflected along its anterior border from the portal vein, underlying connective tissue, and the mesenteric artery, to display a branch of this artery entering the pancreas. By careful blunt dissection, a passage between the pancreas and the mesenteric vein was made along the length of the vein, and two coarse ligatures passed through this passage. The vessels displayed on the medial aspect of the pancreas were ligated with one of these ligatures, and the large portion of pancreas which had been freed from surrounding tissues was thus isolated from the major blood supply. The other ligature was tied around that portion of the pancreas which lies in the angle formed by the mesenteric vein and the bile duct. The pancreatic tissue between these two ligatures was then removed by careful section.

The remainder of the pancreas was approached through an aperture made in the mesentery lying between the duodenum and the omasum. The pancreatic tissue in this site was reflected from the surface of the angle formed by the portal vein and the venous trunk which results from the junction of the left ruminal and the omaso-abomasal veins, and from the surface of this latter trunk. Small vessels leaving the pancreas and entering these veins were ligated and the portion of the pancreas on the medial aspect of the venous trunk was removed. The pancreas was then freed from the omentum overlying the coils of the large intestine, and from the surface of the right ruminal vein. A ligature passed through a passage made by blunt dissection along the lower edge of the mesenteric vein was used to occlude the vessels connecting this portion of the pancreas to the mesenteric vein. Another ligature passed through this passage was tied to enable the pancreas to be severed at this point and to allow the stump of pancreas attached to the mesenteric vein to be removed. The remaining portion of the pancreas consists of a tongue passing under the angle formed by the portal and gastric veins and extending under the posterior vena cava. Ligation of a small vessel leaving this tongue to join the right ruminal vein, allowed this portion of the pancreas to be removed by gentle traction. A careful examination of the site was then made to ensure that no fragments of pancreas remained.

Owing to the highly vascular nature of the pancreas, any small cut or tear led to profuse bleeding. It was therefore necessary to ligate all vessels before cutting, and to prevent unnecessary trauma. The congested appearance of each portion of the pancreas which followed each ligation served to indicate that the blood supply had been satisfactorily occluded, and that this portion could be removed without haemorrhage.

Slight modification of this procedure was sometimes necessary owing to the minor variations in the disposition of the pancreas in the sheep.

METHODS.

Nine adult Merino ewes (1–4 years), and two lambs (1–2 weeks), survived pancreatectomy and were observed for varying periods after the operation.

During the first two weeks following the operation, four of the adults were treated with insulin injected subcutaneously. At the end of this convalescent period these animals were placed in metabolism cages and insulin therapy was discontinued. The diet consisted of equal parts of wheaten and lucerne chaff.

The concentration of sugar and ketone bodies in the blood and the amount of these substances excreted in the urine each day were taken as criteria of the severity of the diabetes. The daily excretion of urinary nitrogen was also determined.

After a period of 1–2 weeks, a submaintenance ration of 100 gm. of the wheaten and lucerne chaff mixture was offered and the effect of the reduced food intake observed.

Two other adults were provided with meadow hay *ad lib.* and were not given insulin at any stage after the operation. Blood samples were taken each day for analyses.

The lambs were confined with their dams in experimental pens and, except when observations were being made, their diabetic state was controlled by subcutaneous injections of insulin.

In order to observe any possible complications due to loss of exocrine pancreatic secretion the pancreatic ducts in eight sheep were ligated and severed and all pancreatic tissue dissected away from the bile duct. Three of these animals were maintained on the same ration as the depancreatized sheep, two on improved clover grass pasture, and three on wheaten chaff or a mixture of wheaten and lucerne chaff. The latter three were pair-fed with three animals which were subjected to similar operative manipulations, but in which the ducts had been left intact.

The total reducing sugar in blood was estimated by the method of Nelson (1944), and Somogyi (1952), the blood ketone bodies by a modification of the method of Behr and Benedict (1926), urinary sugar by the method of Cole (1933), and urinary ketone bodies by the gravimetric method of Van Slyke (Peters and Van Slyke, 1932). Nitrogen in urine was determined by Kjeldahl digestion followed by microtitration in the Pregl apparatus.

RESULTS.

Sixteen animals were pancreatectomized including five which succumbed during the long and difficult operation or within one to two days. Three of the eleven survivors did not become seriously hyperglycaemic and were killed after two months. Histological examinations of tissue taken from the operated area showed that in one of these a small piece of pancreatic tissue had escaped removal. This animal, immediately after the operation, had a blood sugar concentration of 125 mg./100 ml. which fell to 78 mg./100 ml. at the end of five weeks. No pancreatic tissue was found in the other two.

The other eight animals (six adults and two lambs) all developed symptoms of diabetes with blood sugar values about three times normal. Three of the adults, Nos. 64, 49, and 44, became ketonaemic with average blood ketone levels respectively of 21, 20, and 11 mg./100 ml. expressed as total ketone bodies: the other three, Nos. 2, 27, and 77, had average total ketone bodies of 3–4 mg./100 ml. blood. The results from four of the adult sheep uncontrolled with insulin are given in Table 1.

It is evident from these observations that while the hyperglycaemia and glycosuria of the adult depancreatized sheep are diminished when food is restricted, the degree of ketosis is not so closely related to food intake. The excretion of nitrogen in the urine varied between 4·5 and 7 gm. N_2/day when about 500 gm. of fodder were consumed. When the food was limited, however, the urinary nitrogen levels were lowered in those animals (Nos. 2 and 27), which were not ketotic, whereas in the others little change in the rate of nitrogen excretion took place.

TABLE 1.

Depancreatized Sheep—The average values are given together with the range throughout the collection period.

	Sheep	Period in days	Weight kg.	Food intake gm./day	Water intake ml./day	Urine volume ml./day	Blood sugar mg./100 ml.	Urine sugar Total gm./day	Blood ketones mg./100 ml.	Urine ketones Total gm./day	Urine nitrogen Total gm./day
Fed	27	12	19	543 (250-700)	1870 (1000-3500)	1450 (760-2700)	152 (142-178)	33·2 (25·2-36·0)	3·0 (1·9-4·5)	0·23 (0·08-0·55)	7·3 (6·2-8·8)
Fast	27	3	17	100	870 (500-1300)	670 (540-760)	101 (70-125)	3·8 (1·7-5·9)	2·2 (1·9-2·4)	0·10 (0·04-0·15)	5·0 (4·0-6·6)
Fed	49	15	18	548 (450-800)	1420 (780-2700)	800 (740-980)	156 (148-162)	33·2 (28·0-38·3)	10·8 (8·2-13·1)	0·93 (0·70-1·1)	4·6 (3·6-5·5)
Fast	49	5	17	100	1560 (1000-2000)	1290 (550-1900)	133 (108-148)	9·8 (5·3-15·1)	11·5 (9·6-14·0)	0·64 (0·40-0·87)	4·6 (3·9-5·4)
Fed	64	9	28	480 (400-560)	2460 (900-3800)	2380 (1100-3600)	122 (100-132)	36·0 (27·0-46·0)	21·0 (19·0-22·0)	8·6 (3·1-12·0)	6·6 (5·6-7·9)
Fast	64	5	25	100	1500 (800-1700)	1440 (1000-1800)	103 (88-110)	13·6 (8·9-17·6)	24·3 (22·3-27·1)	4·7 (2·5-7·5)	5·5 (4·0-6·8)
Fed	2	20	21·5	560 (200-820)	1850 (1000-3100)	1090 (520-1840)	223 (160-280)	33·0 (19·2-55·7)	3·6 (1·8-5·1)	0·10 (0·07-0·18)	4·5 (1·9-9·9)
Fast	2	6	19·0	100	620 (300-1000)	414 (180-600)	140 (120-183)	5·3 (1·8-10·5)	2·3 (1·4-2·6)	0·11 (0·08-0·15)	2·2 (1·9-2·5)

When insulin was administered to these four animals the amounts required to control the diabetes varied with each animal and with the amount of food eaten: 5 to 10 units of soluble insulin every 12 hours were usually sufficient to lower the blood sugar and blood ketone concentrations to normal levels for most of the time, with little daily loss of sugar or ketone bodies in the urine.

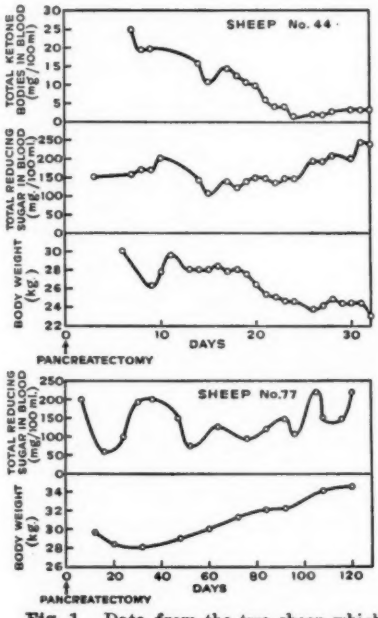

Fig. 1. Data from the two sheep which received no insulin after pancreatectomy.

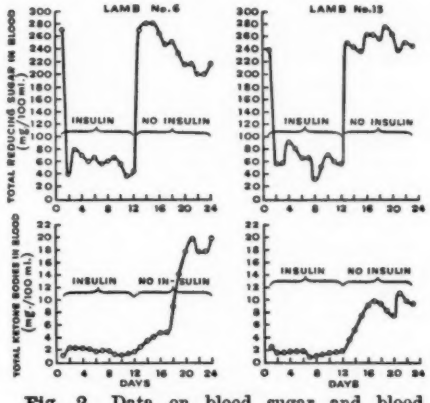

Fig. 2. Data on blood sugar and blood ketone levels in diabetic lambs.

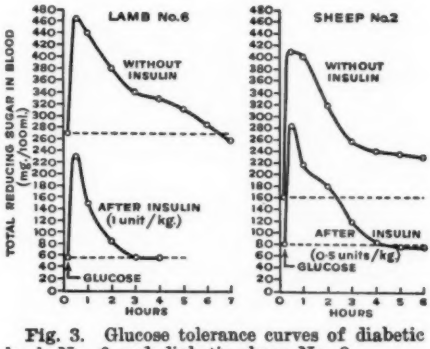

Fig. 3. Glucose tolerance curves of diabetic lamb No. 6 and diabetic sheep No. 2.

Fig. 1 indicates the response of the two sheep (Nos. 44 and 77), which did not receive insulin at any stage after the operation. For about two weeks the severely ketotic animal (No. 44) maintained apparently good condition with little loss of body weight even though the blood sugar concentration was between 110 and 174 mg. p.c. and the blood ketone level between 10 and 26 mg. p.c. The animal then began to lose interest in food and at the same time a reduction in

blood ketones and loss of body weight occurred. Four weeks after the operation the animal's condition deteriorated rapidly and it died in a semi-coma. Autopsy revealed an enlarged and fatty liver which was the only apparent abnormality.

The other animal (No. 77) was observed for more than 5 months during which time it remained hyperglycaemic with a periodic fluctuating blood sugar level but had no marked ketosis. Its weight increased during this period.

Collection of twenty-four hour urine samples from the lambs was not feasible and so blood analyses provided the only quantitative data. Both lambs were hyperglycaemic with blood sugar concentrations of 200 to 280 mg./100 ml. blood, and ketonaemic with concentrations between 8 and 20 mg. ketone bodies/100 ml. blood. Qualitative analyses of urine samples showed a persistent glycosuria and ketonuria. Pertinent data on the lambs are presented in Fig. 2.

Glucose tolerance curves were established for the diabetic adult sheep and lambs with and without insulin treatment. An intravenous injection of a 25 p.c. solution of glucose (1 gm./kg. body weight) was given and the total reducing sugar in the blood determined thirty minutes after the injection and at hourly intervals for 6 to 7 hours. The results indicate the slow rate of return to the pre-injection level typical of the uncontrolled diabetic state (Fig. 3).

The two animals which had a concentration of 3 to 4 mg. of ketone bodies per 100 ml. blood were subjected to careful autopsy immediately after death, and in one of them a small piece of pancreas was detected. Dr. W. Stanley Hartroft, of Washington University, U.S.A., who kindly examined this fragment, reported: "the Beta cells of the islets are grossly pathological. Cytoplasm in these cells is replaced by large vacuolated spaces and specific granules are completely absent. Glycogen stains revealed deposits of stainable glycogen in and about these vacuolations. Deposits of glycogen are also present in the small ductules which likewise appear vacuolated." Thus it is doubtful whether this fragment had retained any considerable part of its normal function.

All animals in which the pancreatic duct was severed maintained good condition for at least a year, and showed comparable weight increase and wool growth with either the sham-operated pair-fed animals or unoperated animals grazing the same pasture. At a post-mortem examination of two of them the pancreas appeared fibrotic, and Dr. Hartroft reported that the pancreatic acinar tissue from one of these did not show very much evidence of atrophy. "There was in this section, considerable, apparently functionable acinar tissue left, and morphologic evidence of the duct ligation was rather difficult to discern." The β cells of the islets were, however, grossly pathological and presented a similar picture to the fragment of tissue reported above.

There was no biochemical evidence, however, that the animals in which the duct had been ligated were in a diabetic condition; blood sugar and blood ketone levels were within the normal range when determined at intervals during the period of observation.

DISCUSSION.

The results indicate that ruminants may suffer severe diabetes after complete removal of the pancreas. The severity of the diabetic syndrome which ensues in sheep appears to be more closely related to the degree of ketosis than to the hyperglycaemia. If the severity of the diabetes is assessed from the concentration of sugar in the blood and the tolerance to injected glucose, the animals after complete pancreatectomy responded in a manner characteristic of diabetic non-ruminants.

If judged primarily by the degree of ketosis, however, half of the adult group developed only mild diabetes. In one of these a small amount of pancreatic tissue was discovered at autopsy (<0·5 p.c.), but histological examination indicated that this piece of tissue was unlikely to produce hormones. Moreover, in the other mildly ketotic cases no pancreatic tissue could be detected.

The reason for the difference in the degree of ketosis which appeared in the adult depancreatized sheep is not clear. The observations on those with mild ketosis agree with reports of the behaviour of goats (Lukens, 1938; Greeley, 1947), calves (Cook, Dye and McCandless, 1949), and sheep (Blomback *et al.*, 1953) after pancreatectomy, in so far as they exhibited none of the severe symptoms usually associated with diabetes in non-ruminants. Those of our sheep which were ketotic, however, did exhibit many of the usual diabetic symptoms.

The amount of insulin required to control the diabetes produced by pancreatectomy is relatively small (5 to 10 units of insulin/day), compared with the insulin requirements (50 to 100 units/day) of sheep made diabetic by alloxan (Jarrett, 1946; McCandless, Woodward and Dye, 1948). The severely ketotic animals were much more difficult to control with insulin than those which developed only a mild ketosis, and often became hypoglycaemic even though only small amounts of insulin were given. Despite careful attention they always lost weight and succumbed within two to three months.

An outstanding feature of the behaviour of the animals which were hyperglycaemic and yet suffered only a mild ketosis, was the fact that the hyperglycaemia could be controlled by very small amounts of insulin. Moreover, they proved capable of maintaining reasonably good condition without any insulin at all, despite the persistent hyperglycaemia.

The reduction in hyperglycaemia which followed the restriction of food indicates some dependence of the blood sugar level on dietary sources. This relationship in conjunction with urinary nitrogen data suggests a low level of gluconeogenesis from protein catabolism in diabetic sheep (McCandless, Woodward and Dye, 1948). While it may be possible to obtain reliable information from urinary nitrogen figures in non-ruminants and in suckling ruminants, conclusions based on data obtained from urinary nitrogen alone may be difficult to interpret in adult ruminants due to the complex reactions associated with the

circulation of nitrogenous compounds in the digestive processes of sheep (Chalmers and Synge, 1954). However, the values obtained from excretion of nitrogen in the urine of the animals in these experiments indicate some difference in the two groups of diabetic animals, but the information available is insufficient to support any definite conclusions at this stage.

Blomback *et al.* (1953) have claimed that loss of body weight by their diabetic sheep was due to protein deficiency following cessation of pancreatic secretion. In the experiments reported here, the body weight and wool growth of those sheep whose pancreatic duct had been ligated and severed were similar to those of normal and sham operated sheep. This indicates that the adult ruminant's capacity to digest and absorb protein is not seriously impaired by lack of pancreatic exocrine secretion.

The association of the α-cells of the pancreas with the degree of hyperglycaemia and ketonaemia is problematical (Volk, Lazarus and Goldner, 1954), and, therefore, from current evidence, it cannot be assumed that changes in the α-cells are responsible for differences between the diabetes induced by pancreatectomy and the diabetes induced by alloxan. The diabetic state produced in sheep by complete extirpation of the pancreas was distinctly different in animals that had been treated similarly: one group of the experimental animals became ketotic and the other did not, and so α-cell influence in alloxan diabetes probably is not concerned with the degree of ketosis.

In view of this difference in degree of ketosis observed in adult depancreatized sheep, together with the fact that animals with mild ketosis are able to exist in a hyperglycaemic state without insulin, and the fact that during this time periodic fluctuations in the blood sugar level may occur, it is suggested that other hormonal influences besides insulin may be implicated in the intermediary metabolism of depancreatized ruminants.

SUMMARY.

Severe diabetes can be produced in adult ruminants by complete removal of the pancreas.

Hyperglycaemia invariably results, but the degree of ketosis varies: some animals become seriously ketotic while others are only mildly ketotic.

The ketotic animals are severely diabetic and usually succumb within 2 months despite insulin therapy. Their insulin requirements, however, are much less than those of alloxan diabetic sheep.

Animals in which the pancreas was left intact and the pancreatic duct severed withstood well the loss of pancreatic exocrine secretion.

It is suggested that other hormonal influences besides insulin may account for variations in the severity of diabetes in depancreatized adult sheep.

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ELECTRON MICROSCOPY OF GLOMERULI IN NEPHROTOXIC SERUM NEPHRITIS

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The advent of the electron microscope, the development of techniques for cutting ultra-thin sections and the improvement in methods of fixation and preparation of tissues for electron microscopic examination have led to new conceptions of the minute structure of the kidney glomerulus (Pease and Baker, 1950; Oberling, Gautier and Bernhard, 1951; Dalton, 1951; Rinehart, Farquhar, Jung and Abul-Haj, 1953; Rinehart, 1955; Hall, 1953, 1954; Reid, 1954; Pease, 1954, 1955; Mueller, Mason and Stout, 1955). Therefore it seems reasonable to apply the electron microscope to the investigation and localization of lesions in the diseased glomerulus.

As human material suitable for electron microscopy of glomeruli could only be obtained by renal biopsy (percutaneous or surgical), experimental investigation offered a simpler alternative. Furthermore, a technique exists for producing diffuse glomerular lesions in animals.

More than 50 years ago Lindemann (1900) produced albuminuria in rabbits injected with serum obtained from the immunization of guinea-pigs with rabbit kidney. Pearce (1904) confirmed Lindemann's findings by using nephrotoxic serum prepared in rabbits and injected into dogs. In addition to albuminuria and cylinduria, he noted thrombosis and degeneration of glomerular tufts.

Since then, many experimenters have produced glomerular changes with nephrotoxic sera and used the technique for varying purposes. Some described the morphology of the kidney lesions (Masugi and Sato, 1934; Schmitt, 1950; Ehrich, Forman and Seifer, 1952; Weinreb, Soules and Wissler, 1954); others investigated the specificity of renal changes (Pearce, 1904; Wilson and Oliver, 1920; Smadel, 1936; Swift and Smadel, 1937), and others were interested in the mechanism of production of the nephritis (Kay, 1940, 1942; Pressman, 1953; Rathe, 1955). Recently Lippman, Marti and Campbell (1952) attempted to correlate glomerular lesions with biochemical changes in the blood, and others to localize or isolate the antigenic components of nephrotoxic sera (Pressman *et al.*, 1948, 1949; Solomon, *et al.*, 1949; Greenspon and Krakower, 1950; Cole, Cromartie and Watson, 1951; Hill and Cruickshank, 1953; Mellors, Siegal and Pressman, 1955).

Glomerular lesions produced by nephrotoxic sera in the rabbit and dog are predominantly inflammatory and proliferative (Masugi and Sato, 1934; Ehrich *et al.*, 1952) but, in the rat, differ according to dose and strength of sera used (Ehrich *et al.*, 1952). Ehrich's observations were, however, disputed by Heymann and Hackel (1953), who thought that apparent differences in the rat were "quantitative rather than qualitative," depending on the time at which the glomeruli were examined.

Florid lesions can also be produced in the mouse. Schmitt (1950) described swollen glomerular loops, narrowed lumina and hyaline thickening of capillary walls, but was not certain which glomerular component participated in the hyaline change.

Likewise, I have never found it easy to localize nodules or thickenings in the glomerulus with the light microscope because of the complexity of loops and the difficulty in distinguishing various components of the capillary walls. On the other hand, the electron microscope shows the glomerular basement membrane and surrounding cells much more distinctly, yet only a few electron micrographs of diseased glomeruli have been published.

Ehrich (1953) illustrated thickening that occurs in the glomeruli of nephrotic rats with an electron micrograph which showed deposits "in or on the inner surface" of the basement membrane and "vesiculation" of the cytoplasm of adjacent epithelial cells. Simer (1954) examined the glomerular capillaries of nephrotic mice with the electron microscope and thought that the basement membrane was thickened and the epithelial pedicels widened and irregular in arrangement. The capillary endothelium was thickened and narrowed the capillary lumina.

Recently I induced lesions in mice with nephrotoxic serum and obtained electron microscopic photographs of the glomerular changes.[1]

Methods.

Production of nephrotoxic serum. Adult male white mice were killed by decapitation, their kidneys removed aseptically and placed in a sterilized container. Sufficient animals (40 to 50) were used to obtain about 20 gm. of kidney (wet weight).

The kidneys were minced coarsely and washed several times with physiological saline (0·85 p.c.) until free from excess blood. After decanting the fluid, physiological saline was added to make a 20 p.c. mixture (weight to volume) which was whisked in an autoclaved blender for 2–3 minutes to obtain a fine suspension. Crystalline penicillin G, 1000 units per ml., was added. A sample was cultured on blood agar for 48 hours and the remainder (if sterile) kept at −20° C. until required.

Antikidney serum was produced in the rabbit by injecting 5 ml. of the kidney suspension intraperitoneally on alternate days for 1 month. After an interval of a week, the rabbit was bled and sufficient blood obtained to give 10–15 ml. of serum. This was heated to 56° C. for 30 minutes (Heymann, 1952) and then stored frozen until ready for use.

Fixation. Under light ether anaesthesia, a kidney was rapidly removed from each mouse required for examination, placed in a drop of 1 p.c. aqueous osmium tetroxide solution (Palade, 1952) and thin slices removed from the cortex. These were cut into pieces about 1 mm. square and left in excess fixative at room temperature for 4 hours.

Dehydration in graded ethyl alcohol solutions and embedding in methacrylate were carried out as suggested by Robertson (1954).

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[1]While this paper was in the press, Piel *et al.* have published electron microscopic observations on nephrotoxic serum nephritis produced in rats with rabbit anti-kidney serum (J. exp. Med., 102, p. 573, 1955). The dosage of serum was very much greater than in the present work. Within 1 hour they found osmiophilic material collected around the pedicel processes; at 6 hours the basement membrane itself became thickened and this persisted for 10 weeks. The very high dosage may perhaps explain this difference from the present findings.

Sections were cut on a rotary Leitz microtome with glass knives, mounted on collodion-covered copper grids and examined with a Philips electron microscope. Relatively low magnifications were used, the resulting negatives being enlarged photographically.

Suitable glomeruli were located in the block by scanning sections approximately 2 μ thick with the light microscope and then cutting thin ones at the appropriate level.

The opposite kidney of each animal was fixed in 4 p.c. formaldehyde, embedded in paraffin wax and section stained with haematoxylin and eosin and Mallory's aniline-blue collagen stain (Cowdry, 1943). A few selected sections were stained with Congo red and crystal violet.

EXPERIMENTAL.

Production of nephritis.

White mice, 16 weeks old, were divided into 4 groups each containing 3 animals.

One group received 0·2 ml. of nephrotoxic serum (N.S.) intraperitoneally on 3 consecutive days and the second 0·1 ml. of N.S. for 3 consecutive days. The third and fourth groups (controls) received 0·1 ml. of saline solution (0·85 p.c.) or 0·1 ml. of normal rabbit serum respectively for 3 consecutive days.

All were given mouse nuts and water *ad lib.* and watched carefully for signs of oedema following the injections.

As no animal appeared sick or oedematous by the 15th day, one (A) which had received 0·2 ml. of N.S. was anaesthetized and kidney material removed. On the 17th day a second mouse (B) from the same group developed a swollen abdomen, and on the 19th day a third (C), which had received 0·1 ml. N.S., was similarly affected. Both had their kidneys sectioned.

Urine from the mice B and C gave a precipitate of protein when boiled or treated with Tsuchiya's reagent (Todd and Sanford, 1948). Although no gravimetric estimations were performed, the protein appeared to be present in greater amounts than in the controls.

In an effort to study the early morphological changes produced in the glomerulus, a small group of 4 mice was injected with 0·2 ml. N.S. and kidney material removed 24 and 48 hours afterwards.

Light microscopy.

Unlike the control animals, the glomerular loops of mice were more cellular than normal except for some areas (in mice B and C) where there was much thickening of capillary walls.

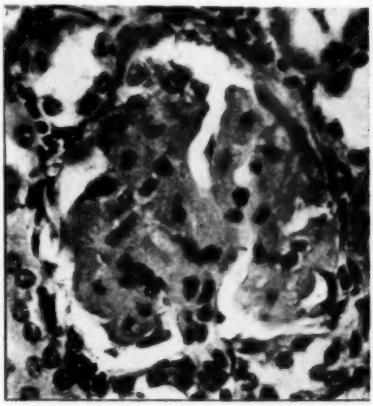


Fig. 1. A mouse glomerulus with most of the loops showing glossy thickening of walls and obliteration of capillary loops. (Haematoxylin and eosin. ×150.)

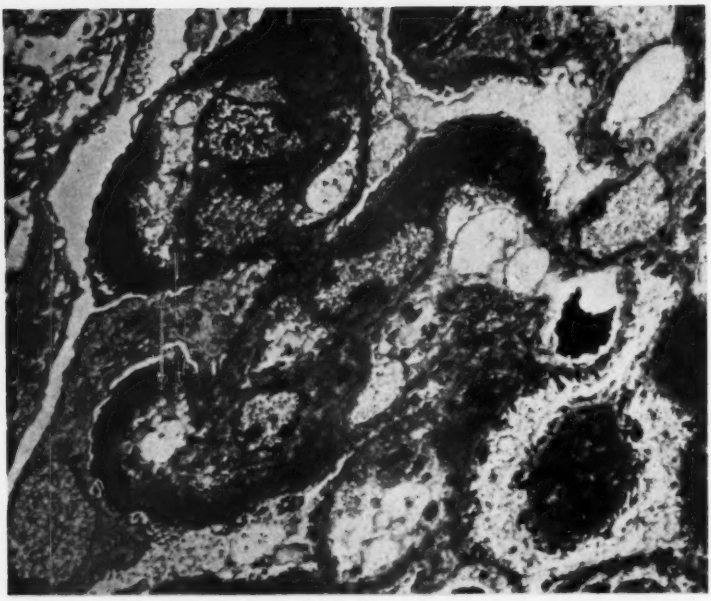


Fig. 2. Segment of mouse glomerulus nineteen days after injection of nephrotoxic serum. Markers represent 10 μ.

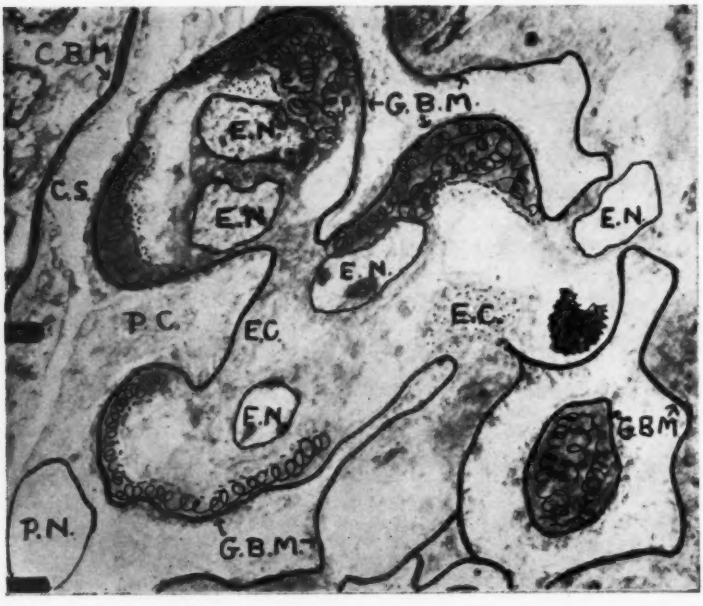


Fig. 3. Illustrative diagram of Fig. 2. Osmic-stained deposits which thicken the loops have been shaded and can be seen encroaching on capillary lumina.

E.N.=endothelial nucleus.
E.C.=endothelial cytoplasm.
G.B.M.=glomerular basement membrane.
C.B.M.=capsular basement membrane.
P.N.=podocyte nucleus.
P.C.=podocyte cytoplasm.
C.S.=capsular space.
R.=red cell.

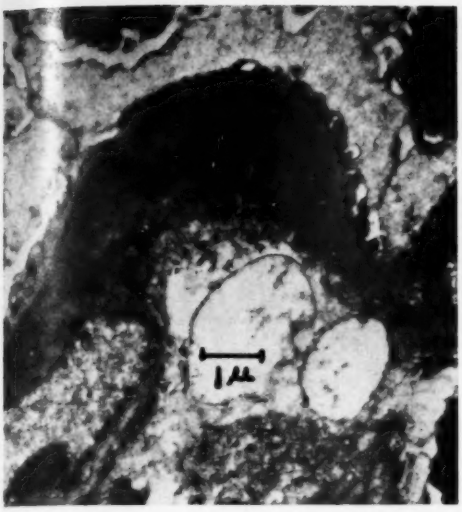
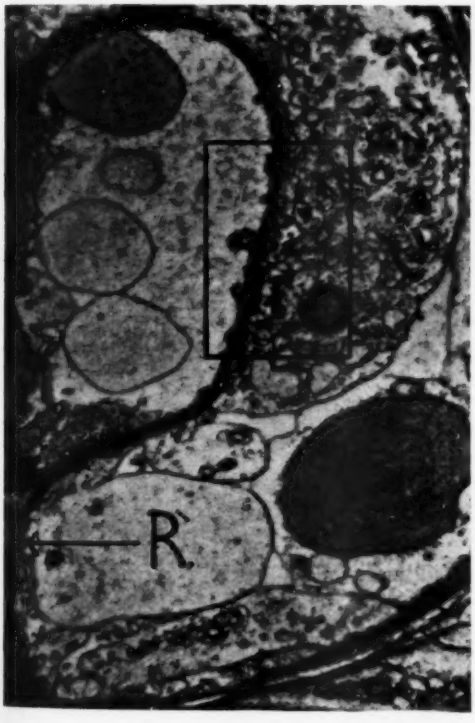


Fig. 4. Enlargement of portion of Fig. 2 showing a glomerular loop. Note the deposits are not homogeneous but contain darker areas. The glomerular basement membrane is visible as a grey line external to, and fenestrated endothelial cytoplasm internal to the deposits. Markers represent 1 μ.



←Fig. 6. Hilar portion of a mouse glomerulus twenty-four hours after nephrotoxic serum. Continuity and reflection of capsular and glomerular basement membrane can be seen at R. Swollen cytoplasm with prominent membrane-lined spaces occurs in the epithelial cell to the right of centre.

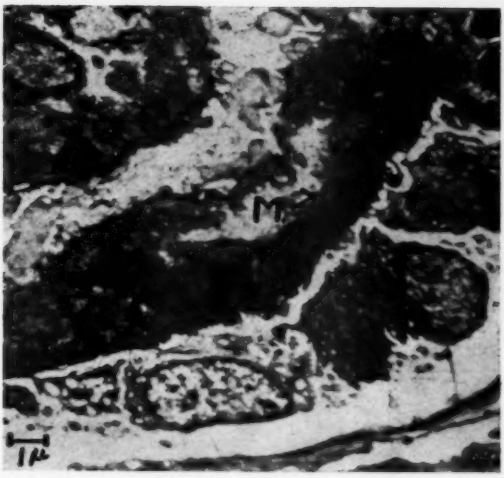


Fig. 5. Periphery of a glomerulus from mouse B showing three vacuolated epithelial cells lying in the capsular space and confluent black masses (M) lying internal to the basement membrane.

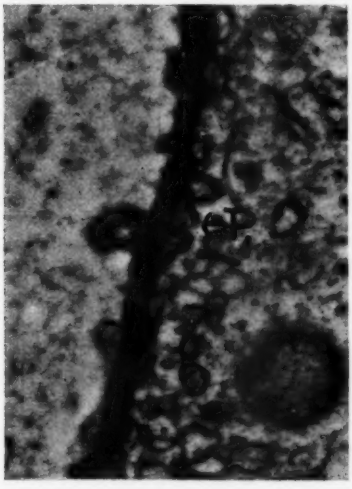


Fig. 7. Enlargement of rectangle in Fig. 6 showing swollen pedicels (P) lying on the outer surface of the glomerular basement membrane. Small protrusions of endothelium occur on the opposite side.

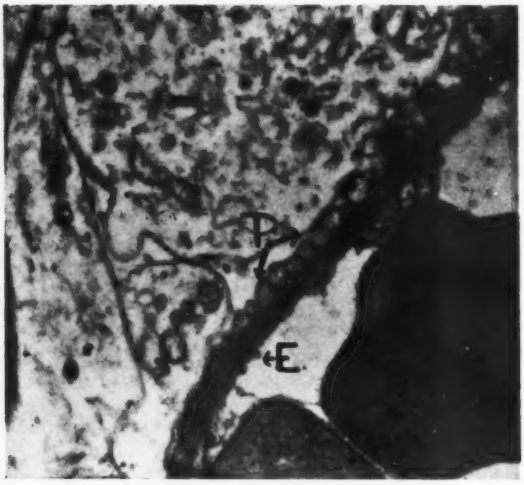


Fig. 8. Another view of closely packed pedicels which are swollen and pale (P). Gaps in the endothelial cytoplasm are visible on the luminal side of the basement membrane (E).

The advanced lesion was characterized by focal or diffuse thickening of capillary loops with refractile, eosin-staining material (Fig. 1). In less affected loops, the material appeared to lie on the inner side of the glomerular basement membrane but with gross capillary narrowing, the membrane blended with the deposits and became indistinguishable from them in haematoxylin and eosin sections.

The glossy material did not occur in the walls of the afferent arterioles nor give positive results when stained for amyloid.

Electron microscopy.

Photographs of abnormal glomeruli were often difficult to interpret because the pathological and degenerative changes (when marked) tended to obscure minute detail.

However, much of the thickening of glomeruli in mice B and C was due to focal accumulations of dark osmic-stained material on the luminal side of the glomerular basement membrane (Figs. 2 and 4). These consisted of varying sized clumps which projected into and narrowed the capillary lumina, as well as thickening the walls. The material did not appear entirely homogeneous, because in thinner sections and tangential views it consisted mainly of irregular black masses (Fig. 5). The basement membrane proper was not thickened.

In many photographs there was a layer of endothelium between the material and the capillary lumen which suggested that the deposits were not thrombi. Thus most deposits appeared to be between the endothelium and basement membrane (Fig. 4). In some areas, however, the endothelium was much thickened and at times seemed to contain dark deposits. The latter effect was thought to be a false impression created by oblique planes of sectioning.

The visceral epithelial cells were undoubtedly swollen and there were many "vacuoles" in the ground substance of the cytoplasm which extended into the peripheral cytoplasm and pedicels (Fig. 6). Although the pedicels were still

recognizable, some were swollen and had lost the relatively dense homogeneous appearance occurring in normal animals.

The altered pedicels were seen best in animals killed 24 or 48 hours after injection of N.S. (Figs. 7 and 8). They were swollen and pale and many had lost the trefoil or inverted "T" appearance where they made contact with glomerular basement membrane.

In addition, there was much less swelling of endothelial cytoplasm and no focal deposits between endothelium and basement membrane in these animals.

DISCUSSION.

The above appearances were consistent with the hypothesis that a pathological "exudate," resulting from the union of antigen and antibody on the glomerular basement membrane, collected between the membrane and endothelial cytoplasm. This supported evidence from other sources that union occurs in the glomerular tuft after injections of nephrotoxic serum and that the basement membrane has antigenic properties (Pressman *et al.*, 1948, 1949; Hill and Cruickshank, 1953).

In addition, the accumulation of material between endothelial cytoplasm and basement membrane lends support to the idea that the two structures are separate entities and that a potential space exists near the membrane in which exudate can collect.

SUMMARY.

The electron microscope was used to study diffuse glomerular lesions produced in mice by the injection of nephrotoxic serum.

The thickened glomerular loops seen with the light microscope appeared to be due, in electron microscopic photographs, to changes in endothelial and epithelial cytoplasm. In advanced lesions, focal osmic-stained deposits appeared mainly between the endothelial cytoplasm and the glomerular basement membrane, the latter not being thickened.

The cytoplasm of the visceral epithelial cells contained many "vacuoles" and the pedicel processes were swollen and pale in comparison with those of normal glomeruli. The latter appearance was seen best in those animals examined before the development of advanced glomerular lesions.

Acknowledgments. I wish to thank Professor J. S. Robertson for helpful criticism and advice during the preparation of this article, and Dr. S. G. Tomlin (Dept. of Biophysics, University of Adelaide) for taking the electron microphotographs.

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THE INTERACTION OF HAEMOGLOBIN WITH SERUM PROTEINS AS SHOWN BY ELECTROPHORESIS

CHANGES OBSERVED IN NEPHRITIC CHILDREN AND DOGS

by MAGDA REICH[1]

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(Accepted for publication 7th December, 1955.)

In the course of a previous investigation on the changes in serum proteins in children with glomerulonephritis, it was noticed that accidentally haemolyzed sera showed three haemoglobin bands on electrophoresis, corresponding in their mobility to the β globulin, a_2 globulin and albumin of the serum. In contrast to this, haemolyzed serum withdrawn from a normal child exhibited only two haemoglobin bands, corresponding to the β globulin and albumin position of the serum. These findings initiated the presented work.

METHOD.

The blood samples used were:

1. Human. (a) 5 normal children aged 5–10 years. (b) 6 children with acute glomerulonephritis aged 3–12 years. (c) A child with chronic glomerulonephritis aged 8 years.

2. Dog. (a) 5 normal mongrels aged 2 months to 3 years. (b) 2 mongrels with Masugi nephritis, early stage, aged 1–2 years. (c) The same dogs as in (b) in the later stage.

For preparation of oxyhaemoglobin solutions heparinized blood was used. After separation of the plasma, the top cellular layer containing leucocytes was removed. The red blood cells were washed with 0·9 p.c. saline three times. They were haemolyzed by freezing and thawing and the resultant solution diluted with 5–8 volumes of veronal buffer pH 8·6. The supernatant separated by centrifugation was analysed spectroscopically. A typical oxyhaemoglobin spectrum was seen.

Mixtures of serum and haemoglobin were prepared by adding one drop of haemoglobin solution to 10 drops of serum. 0·03 ml. of the mixture was used in each estimation.

Filter paper electrophoresis was carried out as described in a previous work (Reich, Coats and McDonald, 1955). Six to seven paper strips were run at the same time, enabling comparison of mobilities under exactly similar conditions.

Strips with haemoglobin only and those containing the serum under analysis were run as controls with each electrophoretic estimation.

Qualitative analysis.

The strips containing the serum plus haemoglobin were sprayed after electrophoresis with a guaiacol-peroxide mixture (Germyn and Thomas, 1954). The strips containing serum only were dyed with bromophenol-blue.

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[1]Aided by a grant from the National Health and Medical Research Council.

Quantitative analysis.

Mixtures of serum plus haemoglobin were prepared by the addition of 0·05 ml. of a 20 p.c. haemoglobin solution to 0·5 ml. of serum. 0·04 ml. of the mixture was applied on each filter paper strip and subjected to electrophoresis. The protein bound haemoglobin areas were cut out from the wet strip and the pigment eluted. The concentration of the eluted haemoglobin was estimated by means of a spectrophotometer, as described by Jermyn and Thomas (1954).

The strips containing serum only were dyed with bromophenol-blue and the concentration of individual protein fractions was estimated by means of an "EEL" Scanner. The areas of the obtained curves were measured planimetrically.

The total serum protein concentration was estimated by the copper sulphate gravimetric method (Phillips *et al.*, 1950).

RESULTS.

Qualitative Results (see Table):

Haemolyzed sera in any of the categories stated in the Table yielded precisely similar results to the prepared mixtures. In every instance the pattern was identical irrespective of the source of haemoglobin added, either normal or nephritic.

TABLE I.

Qualitative Results:

Section I. *The electrophoretic behaviour of excess haemoglobin added to normal and abnormal sera.*

Specimen of Blood	Specimen as prepared for electrophoresis	Number of haemoglobin bands after electrophoresis	Position of bands with respect to the mobility of serum protein fractions	Intensity of bands after spraying
Human:				
Normal	Haemoglobin	One	Human β globulin	Intense
Glomerulonephritis	Haemoglobin	One	Human β globulin	Intense
Normal	Serum plus haemoglobin	Two in 3 cases	1. β globulin 2. α_2 globulin	1. Intense 2. Absent or faint
		Three in 2 cases	3. Albumin	3. Faint
Acute glomerulonephritis	Serum plus haemoglobin	Three	1. β globulin 2. α_2 globulin 3. Albumin	1. Intense 2. Variable 3. Faint
Chronic glomerulonephritis	Serum plus haemoglobin	Three	1. β globulin 2. α_2 globulin 3. Albumin	1. Intense 2. Variable 3. Faint
Dog:				
Normal	Haemoglobin	One	Dog's β globulin	Intense
Masugi nephritis	Haemoglobin	One	Dog's β globulin	Intense
Normal	Serum plus haemoglobin	Two	1. β globulin 2. α_2 globulin	1. Intense 2. Moderate
Masugi nephritis; early stage	Serum plus haemoglobin	Two	1. β globulin 2. α_2 globulin	1. Intense 2. Variable
Masugi nephritis later stage	Serum plus haemoglobin	Two or one (Variable)	1. β globulin 2. α_2 globulin	1. Intense 2. Absent or faint

TABLE 1—*continued.*

Qualitative Results—continued.

Section II. *The effect of varying amounts of haemoglobin on its electrophoretic pattern.*

Specimen of blood	Specimen as prepared for electrophoresis	Number of haemoglobin bands after electrophoresis	Position of bands with respect to the mobility of serum protein fractions	Intensity of bands after spraying
Human:				
Acute glomerulonephritis	Serum plus 1/200 diln. haemoglobin	One	α_2 globulin	Faint
Acute glomerulonephritis	Serum plus 1/150 diln. haemoglobin	One	α_2 globulin	Moderate
Acute glomerulonephritis	Serum plus 1/100 diln. haemoglobin	Three	1. β globulin 2. α_2 globulin 3. Albumin	1. Intense 2. Moderate 3. Faint
Dog:				
Masugi nephritis; early stage	Serum plus 1/200 diln. haemoglobin	One	α_2 globulin	Faint
Masugi nephritis; early stage	Serum plus 1/150 diln. haemoglobin	Two	1. β globulin 2. α_2 globulin	1. Faint 2. Moderate
Masugi nephritis; early stage	Serum plus 1/100 diln. haemoglobin	Two	1. β globulin 2. α_2 globulin	1. Intense 2. Moderate

Samples of the pigment eluted from each of the bands were examined carefully with a hand spectroscope. As obtained, each showed a spectrum typical of oxyhaemoglobin, and on reduction with dithionite, that typical of reduced haemoglobin. The bulk of the pigment associated with the serum proteins was thus normal haemoglobin. There was no evidence for the presence of other pigment derivatives, such as denatured haemoglobin, or compounds of the type of methaemoglobin.

The experiment reported in Section II of the Table suggests that haemoglobin, when added to serum, first combines with the α_2 globulin and only when in excess does it move free or with another protein.

Since α_2 globulin is not a homogenous protein fraction the following experiment was performed to ascertain whether its combination with haemoglobin takes place selectively. Both human and dog sera were used.

The pigmented areas of haemoglobin bound with the α_2 globulin were marked on the strips by pencil. The strips were then dyed with bromophenol-blue. It was seen that the pencilled areas represented the slower moving part of the total α_2 globulin area. This observation was confirmed by comparing the above-mentioned strips with control strips containing only the serum after being dyed with bromophenol-blue.

Experiments were performed to determine the effect of temperature and time on the mobility of the haemoglobin in serum. Mixtures of serum and haemoglobin were kept at 2–6° C. for periods of 5–45 minutes (before electrophoresis) and other mixtures were maintained at 37° C. for similar periods (electrophoresis was performed at 2–4° C. in all cases). These procedures did not affect the behaviour of the haemoglobin.

Quantitative results.

A serum from a child with acute glomerulonephritis was used containing 0·90 gm. p.c. of α_2 globulin and 3·1 gm. p.c. albumin. Five dilutions of this serum were made in veronal buffer (pH 8·6). An excess of haemoglobin was added to each. The optical densities of the eluted haemoglobin bands from the α_2 globulin areas were plotted against the concentration of α_2 globulin in the

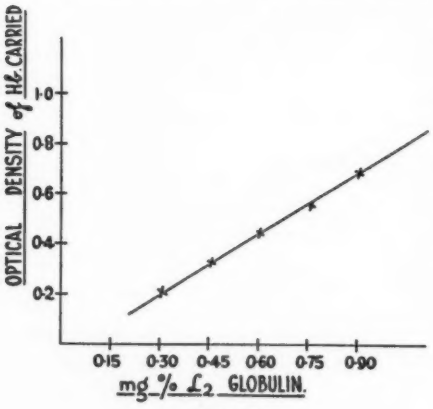

Fig. 1.

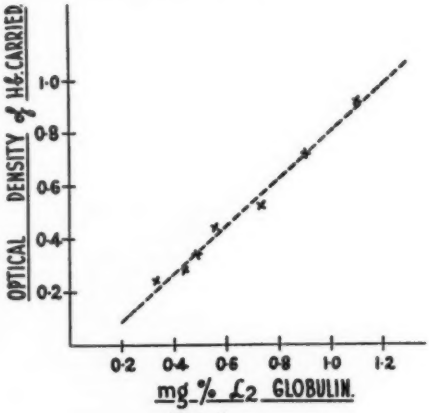

Fig. 2.

serum (Fig. 1). A straight line was obtained. The haemoglobin eluted from the albumin areas had the same optical density 0·27 for concentrations of albumin 3·1, 2·55 and 2·06 gm. p.c. When albumin concentrations of 1·55 and 1·03 gm. p.c. were reached the optical densities were 0·13 and 0·08 respectively.

The above experiment was repeated using dog serum plus haemoglobin. A straight line relationship of the α_2 globulin concentration and the haemoglobin combined with this fraction was found.

Sera from normal children with an α_2 globulin concentration ranging from 0·3 to 0·5 gm. p.c., and nephritic children with an α_2 globulin concentration ranging from 0·6 to 1·1 gm. p.c. were analyzed undiluted. The haemoglobin added was in excess. The concentrations of α_2 globulin of the individual sera were plotted against the optical density of eluted haemoglobin combined with this fraction (Fig. 2). The points obtained are suggestive of a straight line.

DISCUSSION.

It is interesting to note that both human and dog haemoglobins migrate on electrophoresis to a point corresponding to the β globulin of their respective sera. The haemoglobin of both species has an ability to react with proteins of the serum. This is not affected by temperature or time and it may be assumed that this combination is not assisted by enzyme action. In both species the combination of haemoglobin with the α_2 globulin shows a linear relationship to the α_2 globulin level of the serum. Such a relationship does not follow when human haemoglobin reacts with its serum albumin.

Tuttle (1955) reports a haemoglobin reacting substance present in the serum of children, absent in the newborn, migrating with the velocity of α_2 globulin(s). Jayle and Abdellatif (1946) found a protein in the pathological serum, haptoglobin, which forms an enzymic complex with haemoglobin. They proved haptoglobin to be a glycoprotein in nature, and in human and dog's serum it was recovered in the fraction containing α and β globulin, when using the method of alcoholic fractionation of E. S. Cohn. Wieme (1953) observed that the addition of haemoglobin to human serum resulted in a new compound with an electrophoretic mobility between α_2 and β globulin, and this fraction he identifies as Jayle's haptoglobin. No mention of any combination of haemoglobin with the albumin fraction of the serum was made by either of these workers.

It was noticed in the experiments done in this laboratory that the haemoglobin does not combine with the whole of the electrophoretically designated α_2 globulin area. This combination was localized in the slower moving portion of the total area only. Chemical tests and fractionation techniques would be necessary to prove any similarities between the slower moving part of the α_2 globulin fraction which reacts with haemoglobin, and Jayle's haptoglobin.

The difference between normal and nephritic sera in both children and dogs, detectable with the aid of haemoglobin action, was determined by the changes in the α_2 globulin concentration of the serum in this disease. In a previous study (Reich, Coats and McDonald, 1955) it was shown that, in the sera of children with acute glomerulonephritis, there is an increase of α_2 globulin, often as much as three times that of normal value. The same is true for dogs as will be described in a later paper. The results of the experiments described indicate that an increase in the level of α_2 globulins involves a linear increase in the slower moving parts of this fraction. Jayle and Boussier (1954) described haptoglobin, a constituent of α_2 globulins, which increases in concentration in diseases of the collagen system. The haemoglobin itself appears to be unaltered in nephritis, having the same mobility and ability to react with serum proteins as does the haemoglobin of normal human or dog.

In nearly every infectious disease, and many non-infectious ones, there is an increase in the α_2 globulin of the serum (Luetscher, 1947; Bongiovanni and

Wolman, 1949). The ability of haemoglobin to combine with this serum fraction could perhaps be used as a criterion for estimating possible qualitative changes of this protein in the different diseases.

SUMMARY.

The electrophoretic mobility of human and dog haemoglobin was found to be the same as the mobility of β globulin of their respective sera.

The addition of haemoglobin to either normal or nephritic human serum resulted in a combination of haemoglobin with the α_2 globulin and albumin of the serum. The excess haemoglobin remained free and moved with the same mobility as the β globulin of the serum.

In normal and nephritic dogs the haemoglobin added to the serum combined only with the α_2 globulin fraction. The free excess haemoglobin moved with its own mobility corresponding to the β globulin of dog's serum.

In both species the haemoglobin combined with the α_2 globulin was localized in the slower moving part of the total α_2 globulin area.

The combination of haemoglobin with α_2 globulin in human and dog serum showed a linear relationship to the α_2 globulin level of the serum. This relationship held for both normal and nephritic sera in both species.

The reaction of haemoglobin with other serum proteins was not influenced by temperature or time.

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THE USE OF TANNIC ACID TO LINK BLOOD GROUP SUBSTANCES TO GROUP O RED CELLS

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Keogh, North and Warburton (1948) showed that polysaccharide components of some bacteria can be adsorbed to the surface of red cells and that these red cells can be agglutinated specifically by antisera to the bacteria. Boyden (1951) later reported that proteins could also be adsorbed to red cells if the cells were first treated with tannic acid. In this paper it is shown that the A and B blood group substances are not adsorbed to group O red cells as are bacterial polysaccharides, but that they will adhere after preliminary treatment of the cells with tannic acid solution. Such treated cells are agglutinated by and absorb antisera like group AB cells. The mechanism of the tannic acid linkage has been investigated.

Materials and Methods.

Buffered saline. This was prepared by mixing equal volumes of physiological saline and phosphate buffer solution. The buffer solution was obtained by mixing 0·15 M Na_2HPO_4 with 0·15 M KH_2PO_4 in proportions to give the desired pH, either 6·4 or 7·2.

Solutions of blood group substances. Two sources of this material were used.

1. A solution of both A and B substance in isotonic saline is prepared by the Commonwealth Serum Laboratories, Melbourne; three different batches were used. The material was concentrated 20 times by lyophilization and then dialysed against large volumes of buffered saline (pH 6·4) for 60 hours to produce a solution of the blood group substances in buffered saline. The final concentration of the polysaccharide was approximately 2 p.c., but of this three-quarters was B specific and one-quarter A specific (Graydon, J. J., personal communication).

2. A 2 p.c. solution of group A substance was prepared in buffered saline (pH 6·4). The material had been prepared from commercial peptone in 1942 by Dr. V. M. Trikojus, using the method of Goebel (1938).

Tannic acid solution. A solution of one part tannic acid in 20,000 volumes of physiological saline was prepared daily and used only on the day of preparation.

Red blood cells. In all instances the red cells were obtained from blood which had been collected into acid-citrate-dextrose (A.C.D.) solution. The blood had been collected 3 to 10 days previously and the red cells were washed three times with physiological saline before use.

Testing serum. Sera from group A (anti-B), group B (anti-A) and group AB blood donors were used. The anti-D sera were obtained from women who had developed agglutinins of the complete type during pregnancy. All sera were inactivated by heating to 60° C. for five minutes before use.

Iron solution. A 0·15 M solution of ferrous ammonium sulphate ($FeSO_4(NH_4)_2SO_4.6H_2O$) was prepared in distilled water immediately before use. This solution was diluted with

physiological saline (0·15 M) so that the iron concentration of the final solutions was 10 μg./ml. or 20 μg./ml.

Treatment with tannic acid. A 2·5 p.c. suspension of red cells was prepared in buffered saline (pH 7·2) and incubated at 37° C. with an equal volume of tannic acid solution. After 15 minutes the cells were centrifuged and washed once with buffered saline (pH 7·2).

Adsorption of polysaccharide. A 4 p.c. suspension of tannic acid treated cells was prepared in the buffered saline solution of the blood group substances. It was left at room temperature for one hour, or incubated at 37° C. for 15 minutes. The cells were then removed by centrifugation and washed with physiological saline solution containing 0·4 p.c. group AB serum. The cells were finally suspended in this solution. The addition of group AB serum aids in the resuspension of the cells after centrifugation and prevents haemolysis.

Titration of antisera. Serial dilutions of the antisera were prepared using as the diluent physiological saline containing 1 p.c. group AB serum. One drop of each dilution of anti-A and anti-B sera was mixed on a slide with one drop of a 20 p.c. suspension of the red cells. The mixtures were observed for agglutination after five minutes at room temperature. A 1 p.c. suspension of red cells was used for titrating the anti-D agglutinins. The cell-serum mixtures were made in small test tubes which were placed at 37° C. for 30 minutes and then centrifuged. The contents of the tubes were examined microscopically for agglutination.

Nitrogen estimations. These were carried out on the various solutions of the blood group substances by the standard micro-Kjeldahl method.

RESULTS.

Adsorption of group A substance.

Titrations of anti-A serum were performed against group O cells which had been treated with tannic acid and the solution of group A substance. The same serum was also titrated against untreated group O cells, against cells treated with the blood group substance but without tannic acid, and against group AB cells. In all instances the cells were tested against AB serum to exclude non-specific agglutination. The results of a typical experiment are shown in Table 1. It will be seen that the group substance is adsorbed to the red cells only after the cells are treated with tannic acid. Such treated cells agglutinate like group A cells with the antiserum. Similar results were obtained with one of

TABLE 1.

Agglutination of treated and untreated O cells with anti A serum.

Cells	Serum	Serum dilutions							
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Untreated O cells	anti A	−	−	−	−	−	−	−	−
	AB serum	−	−	−	−	−	−	−	−
O cells after treatment with group A substance only	anti A	−	−	−	−	−	−	−	−
	AB serum	−	−	−	−	−	−	−	−
O cells after treatment with tannic acid and group A substance	anti A	+	+	+	+	−	−	−	−
	AB serum	−	−	−	−	−	−	−	−
Group A cells	anti A	+	+	+	+	−	−	−	−
	AB serum	−	−	−	−	−	−	−	−

the three batches of A and B substances from the Commonwealth Serum Laboratories. In this case it was shown that the treated cells were agglutinated by both anti-A and anti-B sera. Attempts to attach A and B substances from the other two batches were not successful, despite all variations of the method.

The adsorption of group A substance to tannic acid treated cells was further demonstrated by showing that coated cells removed anti-A agglutinins from serum. The serum was mixed with equal volumes of treated and untreated cells, incubated at 37° C. for one hour and then titrated against group A cells. The results are shown in Table 2.

TABLE 2.

Absorption of anti A serum by treated and untreated group O cells.

	Serum titres							
	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Unabsorbed serum	+	+	+	+	+	+	−	−
Serum absorbed with cells treated with group A substance only	+	+	+	+	+	−	−	−
Serum absorbed with cells treated with tannic acid and group A substance	−	−	−	−	−	−	−	−

The absorption of agglutinins was shown to be specific, since group O Rh negative cells coated with A and B substances did not absorb anti-D agglutinins.

Action of tannic acid on red cells.

When group AB Rh positive cells were treated with tannic acid the agglutinability of the cells by anti-A, anti-B and anti-D sera was greatly reduced. This is seen in Table 3, which shows the results of titrating these sera against group AB Rh positive cells before and after incubation of the cells with tannic acid solution. It was also shown that cells treated with tannic acid do not absorb

TABLE 3.

Agglutinability of group AB Rh positive cells before and after treatment with tannic acid.

Serum	Type of cells	Serum dilutions									
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Anti A	Untreated	+	+	+	+	+	+	+	+	−	−
	Tannic acid treated	+	+	−	−	−	−	−	−	−	−
Anti B	Untreated	+	+	+	+	+	+	+	−	−	−
	Tannic acid treated	+	+	−	−	−	−	−	−	−	−
Anti D	Untreated	+	+	+	+	+	−	−	−	−	−
	Tannic acid treated	−	−	−	−	−	−	−	−	−	−

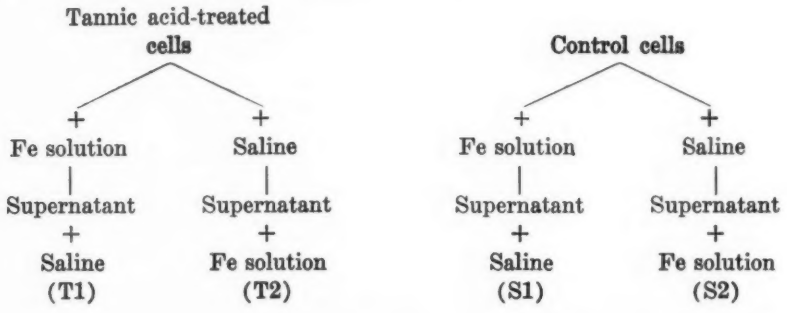
agglutinins as readily as do untreated cells. In one of the experiments performed, untreated group AB cells reduced the titre of an anti-A serum from 1 in 256 to 1 in 4, but after incubation with tannic acid the same volume of cells reduced the titre only to 1 in 32. Similar results were obtained with anti-B and anti-D sera.

The question arose as to whether tannic acid actively removed antigenic material from the red cell surface. The tannic acid solution in which group A cells had been suspended and all the saline used for washing was concentrated by lyophilization to approximately one hundredth of its original volume. This concentrated solution was dialysed against physiological saline and mixed with an equal volume of anti-A serum. The titre of the mixture was found to be the same as that of anti-A serum diluted with an equal volume of saline, indicating that tannic acid does not remove significant amounts of antigenic material from the red cells.

Demonstration of the presence of tannic acid on the red cell surface.

The adhesion of tannic acid to the surface of red cells was demonstrated by showing that tannic acid-treated cells remove iron from a solution of ferrous ammonium sulphate. Iron combines with tannic acid to form insoluble iron tannate.

Group O cells which had been incubated with tannic acid solution were washed six times with physiological saline solution to remove traces of the phosphate buffer. On each occasion approximately 10 volumes of saline were used. The washed cells were divided into equal parts. To one a known volume of the ferrous ammonium sulphate solution was added (T1). An equal volume of saline was added to the other part (T2). The mixtures were left at room temperature for 10 minutes and the cells then removed by centrifuging. The same amount of iron solution was then added to the supernatant of T2 as was originally added to the treated cells (T1). A similar volume of saline was added to the supernatant of T1. A control experiment was performed using the same cells incubated with buffered saline (pH 7·2) without tannic acid. The general plan of the experiment was as follows:—



The iron content of the supernatant solutions was determined by a modification of the method described by Kaldor (1953). Haemoglobin resulting from haemolysis was first precipitated by adding 2 ml. of 4 p.c. trichloracetic acid solution to 8 ml. of the supernatant and heating the mixture to 90° C. for 15 minutes. Any haemoglobin iron not precipitated would be present in the same amount in both T1 and T2; any ferrous ammonium sulphate iron co-precipitated with haemoglobin would also be equal in both solutions. Hence iron removed from solution by the tannic acid-treated cells would be the difference between the iron content of T1 and T2. Non-specific removal of iron by the control cells, probably by small amounts of phosphate buffer, is represented by the difference in iron content of S2 and S1. Therefore the iron removed specifically by tannic acid-treated cells is represented by $(T2—T1)—(S2—S1)$, where the symbols represent the iron content of the corresponding supernatant solutions.

TABLE 4.

Removal of iron from solution by tannic acid treated cells.

Volume of packed tannic acid treated cells	Number of times cells washed after treatment	Volume of Fe solution	Strength of Fe solution	Fe removed from solution (T1-T2)—(S1-S2)
1 ml.	5	10 ml.	20 μg./ml.	16 μg.
1 ml.	5	10 ml.	10 μg./ml.	15 μg.
1 ml.	5	10 ml.	10 μg./ml.	14 μg.
1 ml.	10	10 ml.	10 μg./ml.	16 μg.
1 ml.	5	10 ml.	10 μg./ml.	17 μg.
2 ml.	5	10 ml.	10 μg./ml.	33 μg.

The results are shown in Table 4. It can be seen that iron is removed from solution by the tannic acid-treated cells. The amount is not influenced by the strength of the iron solution used, nor by the number of times the cells are washed. When the volume of cells is double, twice the amount of iron is removed. It would therefore appear that tannic acid adheres to the surface of the red cells.

Nitrogen content of blood group substances.

Nitrogen estimations were performed on two solutions, a preparation from the Commonwealth Serum Laboratories, which failed to adhere to tannic acid-treated cells, and the group A material, which did adhere. The nitrogen content of the dried material, excluding salt, of the Commonwealth Serum Laboratories saline solution was 7·1 p.c., whereas that of the group A material was only 4·8 p.c.

DISCUSSION.

It has long been known that tannic acid may cause agglutination of red cells. In the experiments described in this paper this non-specific agglutination has been prevented by washing and suspending the treated cells in buffered saline (pH 7·2). The nature of the agglutination has been investigated by several workers. Reiner and Fischer (1929) suggested that tannic acid alters the surface molecules of red cells, changing them from a hydrophilic to a hydrophobic state. Freund (1929, 1931) assumed that it produced a change in the surface potential of the cells so that aggregation occurs in the presence of certain electrolytes. Whatever the mechanism of this non-specific agglutination, the results of the present work show that tannic acid becomes attached to the red cell surface.

It is clear from the results reported in this paper that blood group substances can be adsorbed on to tannic acid-treated red cells, but it is not clear why some batches of group A and B substance adhere to tannic acid-treated red cells while other batches do not. It is of interest to note that Boyden used only protein compounds in his original work.

Efforts are being made to determine whether tannic acid-treated cells containing group A and B substances are antigenic in rabbits. It is known that these substances do not produce an antigenic response in rabbits if injected in pure solution, but group A substance will produce potent anti-A agglutinins if conjugated to a foreign protein such as the protein of *Shigella shigae* (Morgan, 1943).

Apart from the action of tannic acid in facilitating the adsorption of proteins, and now certain polysaccharides on to red cells, the specific agglutinability of tannic acid-treated red cells is lowered. This could be due to the removal of antigenic substances from the cells or to a partial masking of the antigens by the tannic acid. Experiments suggest that the latter explanation is more likely, since there is no evidence for the removal of antigenic substances, and there is evidence for the union of tannic acid with the surface of the cell. However, the action of tannic acid is probably more complicated and it may cause some alteration of the structure of the red cell surface.

SUMMARY.

After treatment with tannic acid it was possible to adsorb group A and B substances on to group O cells so that the cells were then agglutinated by and absorbed specific antisera. The agglutinability and absorbing power of group AB Rh positive cells was reduced after treatment with tannic acid. Experiments were performed which indicate that tannic acid becomes attached to red cells.

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